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(54) Title: METHOD OF PREPARING A DOUGH OR A BAKED PRODUCT MADE FROM A DOUGH, WITH ADDITION OF LIPOLYTIC ENZYMES

(57) Abstract: The addition to dough of a combination of two lipolytic enzymes with different substrate specificities produces a synergistic effect on the dough or on a baked product made from the dough, particularlya larger loaf volume of the baked product and/or a better shape retention during baking.

METHOD OF PREPARING A DOUGH OR A BAKED PRODUCT MADE FROM A DOUGH, WITH ADDITION OF LIPOLYTIC ENZYMES

## TECHNICAL FIELD

The present invention relates to methods of preparing a dough or a baked product made from dough by use of lipolytic enzymes, and to compositions for use therein.

## BACKGROUND

WO 94/04035, EP 109244, EP 585988, WO 98/26057, WO 98/45453, WO 99/53769, WO 00/32758 and EP 575133 describe the addition of various lipolytic enzymes to dough in the preparation of bread, e.g. enzymes with activities such as triacylglycerol lipase, phospholipase and galactolipase.

## SUMMARY OF THE INVENTION

The inventors have found that the addition to dough of a combination of two lipolytic enzymes with different substrate specificities produces a synergistic effect on the dough or on a baked product made from the dough, particularly a larger loaf volume of the baked product and/or a better shape retention during baking.

Accordingly, the invention provides a method of preparing a dough or a baked product made from dough, comprising adding a combination of two lipolytic enzymes to the dough. The invention also provides a composition comprising a combination of two lipolytic enzymes.

The combination may comprise at least two lipolytic enzymes selected from the group consisting of galactolipase, phospholipase and triacylglycerol lipase. Thus, the combination may comprise a galactolipase + a phospholipase, a phospholipase + a triacylglycerol lipase or a triacylglycerol lipase + a galactolipase.

## DETAILED DESCRIPTION OF THE INVENTION

### Lipolytic enzyme

The invention uses a combination of lipolytic enzymes, i.e. enzymes which are capable of hydrolyzing carboxylic ester bonds to release carboxylate (EC 3.1.1). The enzyme combination includes at least two of the following three: a galactolipase, a phospholipase and a triacylglycerol lipase, i.e. enzymes predominantly having activity for a galactolipids, a phospholipid, and a triglyceride, respectively. The activities may be determined by any suitable method, e.g. by assays known in the art or described later in this specification.

- Galactolipase activity (EC 3.1.1.26), i.e. hydrolytic activity on carboxylic ester bonds in galactolipids such as DGDG (digalactosyl diglyceride). The

galactolipase activity (digalactosyl diglyceride hydrolyzing activity or DGDGase activity) may be determined, e.g., by the plate assay in this specification or by the monolayer assay 1 or 2 in WO 2000/32758.

- Phospholipase activity (A1 or A2, EC 3.1.1.32 or 3.1.1.4), i.e. hydrolytic activity towards one or both carboxylic ester bonds in phospholipids such as lecithin. The phospholipase activity may be determined by the plate assay in this specification or by an assay WO 2000/32758, e.g. the PHLU, LEU, monolayer or plate assay 1 or 2.
- Triacylglycerol lipase activity (EC 3.1.1.3), i.e. hydrolytic activity for carboxylic ester bonds in triglycerides, e.g. 1,3-specific activity, particularly on long-chain triglycerides such as olive oil. The activity on long-chain triglycerides (olive oil) and short-chain triglycerides (tributyrin) may be determined by the SLU and LU methods (described in WO 00/32758), respectively, or by the plate assay described in this specification. The enzyme may have a substrate specificity for hydrolyzing long-chain fatty acyl groups rather than short-chain groups, expressed e.g. as a high ratio of activities on olive oil and tributyrin, e.g. the ratio SLU/LU. Favorably, this may reduce the development of off-odor in dough containing milk lipids such as butter fat. Suitably, this ratio may be above 3.

Each lipolytic enzyme may have a narrow specificity with activity for one of the three substrates and little or no activity for the other two, or it may have a broader specificity with predominant activity for one substrate and less activity for the other two substrates.

A lipolytic enzyme is considered to be a galactolipase if it has a higher activity on galactolipids than on phospholipids and triglycerides. Similarly, it is considered to be a phospholipase or a triacylglycerol lipase if it has a higher activity for that substrate than for the other two. The comparison may be done, e.g., by the plate assay in this specification using the three substrates; the largest clearing zone indicating the predominant activity.

The enzyme combination may comprise three or more lipolytic enzymes, e.g. comprising a galactolipase, a phospholipase and a triacylglycerol lipase.

The enzyme combination may have low activity on partially hydrolyzed lipids such as digalactosyl monoglyceride (DGMG), lysophospholipids (LPL) and mono- and diglycerides (MG, DG). Favorably, this may lead to accumulation of such partially hydrolyzed lipids in the dough and may improve the properties of the dough and/or the baked product.

**Sources of lipolytic enzymes**

The lipolytic enzymes may be prokaryotic, particularly bacterial, e.g. from *Pseudomonas* or *Bacillus*. Alternatively, the lipolytic enzymes may be eukaryotic, e.g.

from fungal or animal sources. Fungal lipolytic enzymes may be derived, e.g. from the following genera or species: *Thermomyces*, particularly *T. lanuginosus* (also known as *Humicola lanuginosa*); *Humicola*, particularly *H. insolens; Fusarium*, particularly *F. oxysporum*, *F. solani*, and *F. heterosporum; Aspergillus*, particularly *A. tubigensis*, *A. niger*, and *A. oryzae; Rhizomucor; Candida*, particularly *C. antarctica; Penicillium*, particularly *P. camembertii; Rhizopus*, particularly *Rhizopus oryzae;* or *Absidia.*

Some particular examples of lipolytic enzymes follow:

- Phospholipase from bee or snake venom or from mammal pancreas, e.g. porcine pancreas.
- Phospholipase of microbial origin, e.g. from filamentous fungi, yeast or bacteria, such as the genus or species *Aspergillus, A. niger, Dictyostelium, D. discoideum, Mucor, M. javanicus, M. mucedo, M. subtilissimus, Neurospora, N. crassa, Rhizomucor, R. pusillus, Rhizopus, R. arrhizus, R. japonicus, R. stolonifer, Sclerotinia, S. libertiana, Trichophyton, T. rubrum, Whetzelinia, W. sclerotiorum, Bacillus, B. megaterium, B. subtilis, Citrobacter, C. freundii, Enterobacter, E. aerogenes, E. cloacae Edwardsiella, E. tarda, Erwinia, E. herbicola, Escherichia, E. coli, Klebsiella, K. pneumoniae, Proteus, P. vulgaris, Providencia, P. stuartii, Salmonella, S. typhimurium, Serratia, S. liquefasciens, S. marcescens, Shigella, S. flexneri, Streptomyces, S. violeceoruber, Yersinia,* or *Y. enterocolitica.*
- Lipase from *Thermomyces lanuginosus* (*Humicola lanuginosa*) (EP 305216, US 5869438).
- Lipase/phospholipase from *Fusarium oxysporum* (WO 98/26057).
- Lysophospholipases from *Aspergillus niger* and *A. oryzae* (WO 0127251).
- Phospholipase A1 from *Aspergillus oryzae* (EP 575133, JP-A 10-155493).
- Lysophospholipase from *F. venenatum* (WO 00/28044).
- Phospholipase B from *A. oryzae* (US 6146869).
- Lipase from *A. tubigensis* (WO 9845453).
- Lipase from *F. solani* (US 5990069).
- Lipolytic enzyme from *F. culmorum* (US 5830736).
- Phospholipase from *Hyphozyma* (US 6127137).
- Lipolytic enzymes described in PCT/DK 01/00448.
- Lipolytic enzymes described in DK PA 2001 00304.
- A variant obtained by altering the amino acid sequence a lipolytic enzyme, e.g. one of the above, e.g. as described in WO 2000/32758, particularly Examples 4, 5, 6 and 13, such as variants of lipase from *Thermomyces lanuginosus* (also called *Humicola lanuginosa*).

The lipolytic enzymes may have a temperature optimum in the range of 30-90°C, e.g. 30-70°C.

**Synergistic effect**

The combination of the two lipolytic enzymes has a synergistic effect on dough made with the combination or on a baked product made from the dough, particularly improved dough stabilization, i.e. a larger loaf volume of the baked product and/or a better shape retention during baking, particularly in a stressed system, e.g. in the case of over-proofing or over-mixing.

Additionally or alternatively, the synergistic effect on the baked product may include a lower initial firmness and/or a more uniform and fine crumb, improved crumb structure (finer crumb, thinner cell walls, more rounded cells), of the baked product, Additionally or alternatively, there may be a synergistic effect on dough properties, e.g. a less soft dough, higher elasticity, lower extensibility.

Synergy may be determined by making doughs or baked products with addition of the first and the second lipolytic enzyme separately and in combination, and comparing the effects; synergy is indicated when the combination produces a better effect than each enzyme used separately.

The comparison may be made between the combination and each enzyme alone at double dosage (on the basis of enzyme protein or enzyme activity). Thus, synergy may be said to occur if the effect of 0.5 mg of enzyme A + 1.0 mg of enzyme B is greater than the effect with 1.0 mg of enzyme A and also greater than the effect with 2.0 mg of enzyme B.

Alternatively, the comparison may be made with equal total enzyme dosages (as pure enzyme protein). If the effect with the combination is greater than with either enzyme alone, this may be taken as an indication of synergy. As an example, synergy may be said to occur if the effect of 0.5 mg of enzyme A + 1.0 mg of enzyme B is greater than with 1.5 mg of enzyme A or B alone.

Suitable dosages for the enzymes may typically be found in the range 0.01-10 mg of enzyme protein per kg of flour, particularly 0.1-5 mg/kg, e.g. 0.2-1 mg/kg. Suitable dosages for each of the two enzymes in the combination may be found by first determining a suitable dosage for each enzyme alone (e.g. the optimum dosage, i.e. the dosage producing the greatest effect) and using 30-67 % (e.g. 33-50 %, particularly 50 %) of that dosage for each enzyme in the combination. Again, if the effect with the combination is greater than with either enzyme used separately, this is taken as an indication of synergy.

A lipolytic enzyme with phospholipase activity may be used at a dosage of 200-5000 LEU/kg of flour, e.g. 500-2000 LEU/kg. The LEU activity unit is described in WO 99/53769.

A lipolytic enzyme with triacylglycerol lipase activity may be used at a dosage of 20-1000 LU/kg of flour, particularly 50-500 LU/kg. The LU method is described in WO 2000/32758.

**Additional enzyme**

Optionally, an additional enzyme may be used together with the lipolytic enzymes. The additional enzyme may be an amylase, particularly an anti-staling amylase, an amyloglucosidase, a cyclodextrin glucanotransferase, or the additional enzyme may be a peptidase, in particular an exopeptidase, a transglutaminase, a cellulase, a hemicellulase, in particular a pentosanase such as xylanase, a protease, a protein disulfide isomerase, e.g., a protein disulfide isomerase as disclosed in WO 95/00636, a glycosyltransferase, a branching enzyme (1,4-α-glucan branching enzyme), a 4-α-glucanotransferase (dextrin glycosyltransferase) or an oxidoreductase, e.g., a peroxidase, a laccase, a glucose oxidase, a pyranose oxidase, a lipoxygenase, an L-amino acid oxidase or a carbohydrate oxidase.

The additional enzyme may be of any origin, including mammalian and plant, and preferably of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

The amylase may be a fungal or bacterial alpha-amylase, e.g. from *Bacillus*, particularly *B. licheniformis* or *B. amyloliquefaciens*, or from *Aspergillus*, particularly *A. oryzae*, a beta-amylase, e.g. from plant (e.g. soy bean) or from microbial sources (e.g. *Bacillus*).

The xylanase is preferably of microbial origin, e.g. derived from a bacterium or fungus, such as a strain of *Aspergillus*, in particular of *A. aculeatus*, *A. niger* (cf. WO 91/19782), *A. awamori* (WO 91/18977), or *A. tubigensis* (WO 92/01793), from a strain of *Trichoderma*, e.g. *T. reesei*, or from a strain of *Humicola*, e.g. *H. insolens* (WO 92/17573).

The amyloglucosidase may be from *Aspergillus*, particularly *A. oryzae*.

The glucose oxidase may be a fungal glucose oxidase, particularly from *Aspergillus niger*.

The protease may be a neutral protease from *Bacillus amyloliquefaciens*.

**Anti-staling amylase**

The method or the composition of the invention may include addition of an anti-staling amylase. In particular, a galactolipase and a phospholipase may be used together with an anti-staling amylase, as described in WO 99/53769. The anti-staling amylase is an amylase that is effective in retarding the staling (crumb firming) of baked products, particularly a maltogenic alpha-amylase, e.g. from *Bacillus stearothermophilus* strain NCIB 11837.

Alternatively, the method or composition of the invention may be made without addition of an anti-staling amylase. In particular, a lipase and a phospholipase may be used without addition of an anti-staling amylase or without addition of a maltogenic alpha-amylase.

**Composition comprising lipolytic enzymes**

The present invention provides a composition comprising a combination of two lipolytic enzymes as described above. The composition may be an enzyme preparation for use as a baking additive. The composition may also comprise flour and may be a dough or a premix.

**Enzyme preparation**

The composition may be an enzyme preparation comprising a combination of lipolytic enzymes, for use as a baking additive in the process of the invention. The enzyme preparation may particularly be in the form of a granulate or agglomerated powder, e.g. with a narrow particle size distribution with more than 95 % (by weight) of the particles in the range from 25 to 500 μm.

Granulates and agglomerated powders may be prepared by conventional methods, e.g. by spraying the enzymes onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, e.g. a salt (such as NaCl or sodium sulfate), a sugar (such as sucrose or lactose), a sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy.

The enzyme preparation may also be supplied as a liquid formulation, particularly a stabilized liquid. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods.

**Dough**

The dough of the invention generally comprises wheat meal or wheat flour and/or other types of meal, flour or starch such as corn flour, corn starch, rye meal, rye flour, oat flour, oat meal, soy flour, sorghum meal, sorghum flour, potato meal, potato flour or potato starch.

The dough of the invention may be fresh, frozen or par-baked.

The dough of the invention is normally a leavened dough or a dough to be subjected to leavening. The dough may be leavened in various ways, such as by adding chemical leavening agents, e.g., sodium bicarbonate or by adding a leaven (fermenting dough), but it is preferred to leaven the dough by adding a suitable yeast culture, such as a culture of *Saccharomyces cerevisiae* (baker's yeast), e.g. a commercially available strain of *S. cerevisiae*.

The dough may also comprise other conventional dough ingredients, e.g.: proteins, such as milk powder, gluten, and soy; eggs (either whole eggs, egg yolks or egg whites); an oxidant such as ascorbic acid, potassium bromate, potassium iodate, azodicarbonamide (ADA) or ammonium persulfate; an amino acid such as L-cysteine; a sugar; a salt such as sodium chloride, calcium acetate, sodium sulfate or calcium sulfate.

The dough may comprise fat (triglyceride) such as granulated fat or shortening, but the invention is particularly applicable to a dough where less than 1 % by weight of fat (triglyceride) is added, and particularly to a dough which is made without addition of fat.

The dough may further comprise an emulsifier such as mono- or diglycerides, diacetyl tartaric acid esters of mono- or diglycerides, sugar esters of fatty acids, polyglycerol esters of fatty acids, lactic acid esters of monoglycerides, acetic acid esters of monoglycerides, polyoxyethylene stearates, or lysolecithin.

**Pre-mix**

The invention also provides a pre-mix comprising flour together with two lipolytic enzymes as described above. The pre-mix may contain other dough-improving and/or bread-improving additives, e.g. any of the additives, including enzymes, mentioned above.

## MATERIALS AND METHODS

### Enzyme activity assays

Phospholipase activity (PHLU)

Phospholipase activity is measured as the release of free fatty acids from lecithin. 500 µl 4% L-alpha-phosphatidylcholine (plant lecithin from Avanti), 5 mM $CaCl_2$ in 50 mM NaOAc, pH 5 is added to 50 µl enzyme solution diluted to an appropriate concentration in water. The samples are incubated for 10 min at 30 °C and the reaction stopped at 95 °C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids are determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture is added 250 µl Reagent A and incubated 10 min at 37 °C. Then 500 µl Reagent B is added and the sample is incubated again, 10 min at 37 °C. The absorption is measured at 550 nm. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95 °C) + substrate) are included. Oleic acid is used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min at these conditions.

Plate assay for phospholipase activity

A) 50 ml 2% agarose in purified water is melted/stirred for 5 minutes and cooled to 60 - 63°C.

B) 50 ml 2% plant L-alpha-Phosphatidylcholine 95% in 0,2M NaOAc, 10 mM $CaCl_2$, pH 5,5 at 60°C in 30 min. is blended in 15 sec. with ultrathorax.

Equal volumes of 2% agarose and 2% Lecithin (A and B) are mixed. 250 µl 4 mg/ml crystal violet in purified water is added as indicator. The mixture is poured into appropriate petri dishes (e.g. 30 ml in 14cm Ø dish), and appropriate holes are made in the agar (3-5 mm) for application of enzyme solution.

The enzyme sample is diluted to a concentration corresponding to $OD_{280}$ = 0.5 and 10 microliter is applied into holes in the agarose/lecithin-matrix. Plates are incubated at 30°C and reaction zones in the plates are identified after 20-24 hours incubation, and the size of the clearing zone indicates the phospholipase activity.

Plate assays for galactolipase and triacylglycerol lipase activity

Plate assays are carried out as for the phospholipase assay, except that digalactosyl diglyceride (DGDG) or olive oil is used instead of L-alpha-Phosphatidylcholine.

**Baking methods**

Sponge dough

A liquid sponge is prepared by mixing 34.8 parts of water, 60 parts of flour and 1.5 parts of instant yeast, and fermenting for 3 hours at 24°C. A dough is then prepared by mixing the liquid sponge with 22.93 parts of water, 40 parts of flour, 0.5 part of instant yeast, 11.26 parts of 42 high-fructose corn syrup, 0.25 part of calcium propionate, 2 parts of oil and 2 parts of salt, 50 ppm of ascorbic acid 50 parts of wheat flour, 0.5 part of SSL (sodium stearoyl-2-lactylate), 2 parts of salt, 6 parts of sugar and water and ascorbic acid as required.

European straight dough procedure

A dough is prepared by mixing 100 parts (by weight) of wheat flour, 4 parts of yeast, 1.5 parts of salt and 1.5 parts of sugar with water and ascorbic acid as required to reach a suitable dough consistency.

Shape factor (shape retention)

The shape factor is taken as the ratio between the height and diameter of rolls after baking (average of 10 rolls). A higher value indicates a better shape retention.

Dough softness

Softness is a measure of the degree to which, or ease with which, a dough will compress or resist compression. A sensory evaluation is done by a trained and skilled baker feeling and squeezing the dough. The results are expressed on a scale from 0 (little softness) to 10 (very soft) with the control (dough without enzyme addition) taken as 5.

Dough extensibility

Extensibility is a measure of the degree by which a dough can be stretched without tearing. A sensory evaluation is done by a trained and skilled baker pulling a piece of kneaded dough (about 30 g) and judging the extensibility. The results are expressed on a scale from 0 (Short /low extensibility) to 10 (long /high extensibility) with the control (dough without enzyme addition) taken as 5.

Dough elasticity

Elasticity is a measure of the degree to which a dough tends to recover its original shape after release from a deforming force. It is evaluated by rolling a piece of dough (about 30 g) to a size of about 10 cm, and having a trained and skilled baker carefully pulling at opposite ends to judge the resistance and elasticity. The results are expressed on a scale from 0 (low/weak elasticity/recovery) to 10 (high/strong elasticity/recovery) with the control (dough without enzyme addition) taken as 5.

## EXAMPLES

### Example 1: Synergistic effect of phospholipase and galactolipase on dough stabilization

Lipolytic enzyme combinations were tested in a European Straight dough procedure as described above. Fungal alpha-amylase (Fungamyl Super MA, 40 ppm) and an oxidation system (ascorbic acid, 30 ppm) were added to the dough system. Each dough was split into rolls and pan bread. Over-proofing (indicating a stressed system) was carried out for the rolls (70 min.) and the pan bread (80 min.).

Combinations with the following lipolytic enzymes were tested: Variant 39 was tested in combination with variant 91 or with *Aspergillus oryzae* phospholipase. Variants 39 and 91 are variants of the *Thermomyces lanuginosus* lipase according to WO 2000/32758.

The combination of variants 91 and 39 was selected because of the high phospholipase activity and the high galactolipase activity, respectively. The *Aspergillus oryzae* phospholipase and variant 39 combination were chosen due to the combination of a pure phospholipase and an enzyme with high DGDG activity. The plate assays

described above showed that each enzyme wasx specific with little or no activity for the two other substrates.

The lipolytic enzymes were added according to the table below. The tests with a single enzyme were conducted with a dosage found to be optimum for the enzyme in question, and combinations were tested as indicated, with each enzyme at 33, 50 or 67 % of optimal dosage.

| Lipolytic enzyme | Rolls | | Pan bread |
|---|---|---|---|
| | Specific volume (ml/g) | Shape factor | Specific volume (ml/g) |
| Variant 91 (20 LU/kg) | 7.52 | 0.66 | 5.75 |
| Variant 39 (250 LU/kg) | 7.42 | 0.65 | 5.77 |
| Variant 91 (50 %) + variant 39 (33%) | 7.57 | 0.66 | 5.94 |

The results demonstrate that the combination of Variant 91 with Variant 39, added at 50 % and 33 % respectively of optimal dosage, improves the specific volume for both the rolls and the pan bread compared to the each enzyme added separately at optimum dosage.

The results regarding volume and stability improvement from the combination of *Aspergillus oryzae* phospholipase with Variant 39 are listed in the table below.

| Lipolytic enzyme | Rolls | | Pan bread |
|---|---|---|---|
| | Specific volume (ml/g) | Shape factor | Specific volume (ml/g) |
| *A. oryzae* Phospholipase 0,1 mg/kg | 6,27 | 0,57 | 4,96 |
| Variant 39 (250 LU/kg) | 6,40 | 0,60 | 5,18 |
| *A. oryzae* Phospholipase (33 %) + variant 39 (67%) | 7,31 | 0,68 | 5,80 |

The combination of *A. oryzae* Phospholipase and Variant 39 at 33 % and 67 %, respectively, of optimal dosage increases the specific volume considerably

compared to each enzyme added separately at optimum dosage. The combination also has a positive contribution to the shape factor of the rolls.

Both the results described above show that the combination of a phospholipase and a galactolipase improves the volume and stability (shape factor) of the rolls and bread, compared to the rolls and bread containing up to thrice the dosages of the enzymes added separately.

**Example 2: Synergistic effect of triacylglycerol lipase and phospholipase on dough stabilization**

A phospholipase A2 from porcine pancreas was tested in combination with a 1,3-specific triacylglycerol lipase from *Thermomyces lanuginosus* in the European straight dough procedure as described above. The results were compared to each enzyme used alone in dosages considered to be optimal. The enzyme combination was made with 50% of optimal dosage of each of the enzymes.

Each dough was split into rolls and pan bread. The rolls were proofed for 70 minutes (over proofing), and the pan bread was proofed for 80 minutes (over proofing). The over proofing was carried out to stress the system in order to test the stabilizing effect of the enzymes.

| | Rolls | | Pan bread |
|---|---|---|---|
| | **Sp. Vol (ml/g)** | **Shape factor** | **Sp. Vol (ml/g)** |
| **Phospholipase (3mg)** | 6.24 | 0.56 | 5.60 |
| **Triacylglycerol lipase (1000LU)** | 6.43 | 0.58 | 5.43 |
| **Phospholipase + triacylglycerol lipase (50%/50%)** | 6.88 | 0.60 | 5.93 |

The two enzymes were found to be very specific, i.e. the triacylglycerol lipase has very little activity on phospholipid and galactolipids, and the phospholipase has very little activity on triglycerides and galactolipids.

The results show that when the phospholipase and the triacylglycerol lipase are combined they give a better volume and shape factor than each of the enzyme separately in a stressed system.

**Example 3: Synergistic effect on dough properties and loaf volume**

The combination of Lipase/phospholipase from *Fusarium oxysporum* (FoL) and Variant 6 on dough and bread was evaluated. Variant 6 is a variant of the

*Thermomyces lanuginosus* lipase with the following amino acid alterations (SPIRR indicates a peptide extension at the N-terminal, and 270AGGFS indicates a peptide extension at the C-terminal).

- Variant 6: SPIRR +G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270AGGFS

Loaves were prepared according to the invention by adding Variant 6 (25 LU/kg flour) and FoL (500 LU/kg flour) to the dough. For comparison, loaves were baked without lipolytic enzymes, with FoL alone (1000 LU/kg) or Variant 6 alone (50 LU/kg) which were found to be the optimal dosages for the enzymes. The LU assay method is described in WO 2000/32758.

The standard sponge dough WPB formula was used as described above, with the hydrated distilled MG and SSL eliminated to avoid masking effects on the enzyme. Loaves contained 2% soy oil as well as fungal amylase and pentosanase (Fungamyl Super MA, 50 ppm). The oxidation system was 50 ppm ascorbic acid. In addition to subjective evaluations, crumb softness and elasticity were measured 24 hours after baking. The trial was repeated once.

Dough evaluations. Evaluations of the dough at the sheeter are shown below. The dough scores for the two trials were identical.

| Lipolytic enzyme | None | FoL | Variant 6 | FoL + Variant 6 |
|---|---|---|---|---|
| Softness | 5.5 | 4.5 | 4.5 | 4.0 |
| Extensibility | 5.0 | 4.5 | 4.5 | 4.0 |
| Elasticity | 5.0 | 5.5 | 5.5 | 6.0 |

The results show that the combination of FoL and Variant 6 yielded dough that was less soft, less extensible and more elastic than either enzyme alone at double dosage.

The specific volume data from the tested loaves are shown below. Reproducibility between the 2 days was high.

| **Lipolytic enzyme** | **None** | **FoL** | **Variant 6** | **FoL + Variant 6** |
|---|---|---|---|---|
| **Specific Volume, cc/gram** | 6.0 | 6.15 | 6.15 | 6.3 |

The results demonstrate that the combination of two lipolytic enzymes gives a larger loaf volume than either enzyme alone at double dosage.

**Example 4: Synergistic effect on dough stabilization.**

Variant 32 was tested in combination with Variant 13 and Variant 60. The variants are variants of the *Thermomyces lanuginosus* lipase with the following amino acid alterations (where SPIRR indicates a peptide extension at the N-terminal, and 270AGGFS indicates a peptide extension at the C-terminal):

- Variant 32: 91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N + 270AGGFS
- Variant 60: G91A +D96W +E99K +G263Q +L264A +I265T +G266S +T267A +L269N + 270AGGFS
- Variant 13: D96F +G266S

Each combination was tested in a European straight dough procedure, as described above. The results were compared to each enzyme used alone. Lipolytic enzymes were added as shown in the table below. The tests with a single enzyme were conducted with a dosage considered optimum for that enzyme, and the enzyme combinations were tested with each enzyme at 50 % of the optimum dosage. The combination of Variant 32 and Variant 13 was also tested with each enzyme at 40 % of the optimum dosage, i.e. 20 % lower dosage.

Each dough was split into rolls and pan bread. The rolls were proofed for 70 minutes (over proofing), and the pan bread was proofed for 80 minutes (over proofing). The over proofing was carried out to stress the system, in order to test the lipolytic enzymes as stabilizers.

The results from over-proofing are shown below:

| Lipolytic enzyme added | Rolls | | Pan bread |
|---|---|---|---|
| | Sp. Vol. (ml/g) | Shape factor | Sp. Vol. (ml/g) |
| | 70 min | 70 min | 80 min |
| Variant 32 (200LU) | 7.78 | 0.67 | 6.16 |
| Variant 13 (500LU) | 7.24 | 0.66 | 5.69 |
| Variant 60 (100LU) | 7.02 | 0.67 | 5.69 |
| Variant 32+Variant 13, 50% | 8.26 | 0.71 | 6.24 |

| | | | |
|---|---|---|---|
| **Variant 32+Variant 13, 40%** | 8.03 | 0.69 | 6.30 |
| **Variant 32+Variant 60, 50%** | 7.87 | 0.69 | 6.39 |

The results show that when the bread is stressed (over-proofed), the combinations of Variant 32 with Variant 13 or Variant 60 clearly give an improved volume and shape compared to each enzyme used alone, particularly Variant 32 + Variant 13, even at reduced dosage. The results reveal that when bread is stressed (over proofed), the combinations show a significantly improved effect on volume and shape factor.

Furthermore, it was observed that the combination of Variant 32 and Variant 13 at 40 % of optimum dosage provided a more uniform and fine crumb compared to each enzyme used alone.

## CLAIMS

1. A method for increasing the loaf volume or improving the shape retention of a baked product made from dough, comprising adding to the dough:

a) a phospholipase and a galactolipase, or

b) a phospholipase and a triacylglycerol lipase, or

c) a galactolipase and a triacylglycerol lipase.

2. A method of preparing a dough or a baked product made from dough, comprising adding to the dough:

a) a galactolipase and

b) a phospholipase or a triacylglycerol lipase.

3. A method of preparing a dough or a baked product made from dough, comprising adding to the dough a combination of two lipolytic enzymes wherein the combination produces a synergistic effect on the loaf volume or shape retention of the baked product.

4. The method of the preceding claim wherein the two lipolytic enzymes are :

a) a phospholipase and a galactolipase, or

b) a phospholipase and a triacylglycerol lipase, or

c) a galactolipase and a triacylglycerol lipase.

5. A method of preparing a dough or a baked product made from dough, which comprises adding to the dough:

a) a phospholipase and a galactolipase, or

b) a phospholipase and a triacylglycerol lipase, or

c) a galactolipase and a triacylglycerol lipase

and which does not comprise adding a maltogenic alpha-amylase to the dough.

6. A composition comprising:

a) a galactolipase and

b) a phospholipase or a triacylglycerol lipase.

7. A composition comprising a combination of two lipolytic enzymes wherein the combination produces a synergistic effect on the loaf volume or shape retention of a baked product made from dough including the composition.

8. The composition of the preceding claim wherein the two lipolytic enzymes are:

a) a phospholipase and a galactolipase, or

b) a phospholipase and a triacylglycerol lipase, or

c) a galactolipase and a triacylglycerol lipase.

9. A composition which comprises.

a) a phospholipase and a galactolipase, or

b) a phospholipase and a triacylglycerol lipase, or

c) a galactolipase and a triacylglycerol lipase,

and which does not comprise a maltogenic alpha-amylase.

10. The composition of any preceding claim which comprises flour, particularly a dough or a premix.

11. A method of preparing a dough or a baked product made from dough, comprising:

a) determining substrate specificities of at least two lipolytic enzymes,

b) selecting two lipolytic enzymes which are:

i) a phospholipase and a galactolipase, or

ii) a phospholipase and a triacylglycerol lipase, or

iii) a galactolipase and a triacylglycerol lipase.

c) for each enzyme, determining an effective dosage in the dough to increase the loaf volume or improve the shape retention of a baked product made from the dough,

d) adding to the dough a combination of the first and the second lipolytic enzyme wherein each enzyme is added in an amount of 33-67 of the effective dosage.

12. A method for producing a lipolytic enzyme preparation, comprising:

a) determining the substrate specificities of at least two lipolytic enzymes,

b) selecting two lipolytic enzymes which are:

i) a phospholipase and a galactolipase, or

ii) a phospholipase and a triacylglycerol lipase, or

iii) a galactolipase and a triacylglycerol lipase.

c) making baked products from doughs with addition of the two lipolytic enzymes separately and in combination,

d) determining the loaf volumes or the shape retention of the baked products,

e) selecting two lipolytic enzymes having a synergistic effect, and

f) producing the enzyme preparation comprising a combination of the two lipolytic enzymes.

13. A method of preparing a dough or a baked product made from dough, comprising:

a) selecting two lipolytic enzymes,

b) for each enzyme, determining an effective dosage in the dough to increase the loaf volume or improve the shape retention of a baked product made from the dough,

c) adding to the dough a combination of the first and the second lipolytic enzyme wherein each enzyme is added in an amount of 33-67 % of the effective dosage.

14. The method of the preceding claim wherein the two lipolytic enzymes are:

a) a phospholipase and a galactolipase, or

b) a phospholipase and a triacylglycerol lipase, or

c) a galactolipase and a triacylglycerol lipase.

# INTERNATIONAL SEARCH REPORT

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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, EPO-Internal, PAJ, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
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[X] Further documents are listed in the continuation of box C.

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(54) Title: LIPOLYTIC ENZYME VARIANTS

(57) Abstract

The substrate specificity of a lipolytic enzyme can be modified by making alterations to the amino acid sequence in a defined region of the lipolytic enzyme, so as to increase the level of a desired activity or to decrease the level of an undesired activity. Thus, the inventors have developed lipolytic enzyme variants with a modified amino acid sequence with a substrate specificity which can be tailored for specific uses.

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# LIPOLYTIC ENZYME VARIANTS

## FIELD OF THE INVENTION

The present invention relates to a method of altering the substrate specificity of a lipolytic enzyme by modifying the amino acid sequence, and to lipolytic enzyme variants obtained by such modification. The invention also relates to a screening method for lipolytic enzymes.

## BACKGROUND OF THE INVENTION

Lipolytic enzymes (such as lipases and phospholipases) are capable of hydrolyzing carboxylic ester bonds in a substrate to release carboxylic acids. The hydrolytic activity on different ester bonds is important for the usefulness of the lipolytic enzyme in various industrial applications.

Thus, enzymes with a high phospholipase activity are useful in a wide range of applications such as baking (US 4,567,046), filtration of wheat starch hydrolysate (US 5,264,367) and treatment of vegetable oil to reduce the content of phospholipid (US 5,264,367). For the treatment of vegetable oil, the enzyme should have a low lipase activity, i.e. a low hydrolytic activity towards ester bonds in triglycerides..

WO 98/45453 indicates that an enzyme with a high hydrolytic activity on digalactosyl diglyceride (DGDG) is useful in baking.

It is well known to add a lipase to laundry detergents to aid in the removal of greasy soils (e.g. EP 258,068).

The release of short-chain fatty acids as free fatty acids (FFA) may be desirable for flavor development in food products, e.g. in cheese ripening (M. Hanson, ZFL, 41 (10), 664-666 (1990)).

The three-dimensional (3D) structure of several lipolytic enzymes is known, and several structures are known to contain a so-called "lid" which may be in an open or closed state covering the active site. Brady et al., Nature, 343, 767-770 (1990). Brzozowski A M et al., Nature, 351, 491 (1991). Derewenda et al., Biochemistry, 31 (5), 1532-1541 (1992).

F. Hara et al., JAOCS, 74 (9), 1129-32 (1997) indicates that some lipases have a certain phospholipase activity, whereas most lipases have little or no activity on phospholipids. Thus, phospholipase activity has been described in the lipases from guinea pig pancreas, *Fusarium oxysporum* and *Staphylococcus hyicus*, and attempts have been made to relate the phospholipase activity to the structure of the

lipase. WO 98/26057; M.D. van Kampen et al., Chemistry and Physics of Lipids, 93 (1998), 39-45; A. Hjorth et al., Bio- chemistry 1993, 32, 4702-4707.

The prior art has described the effect on chain-length selectivity by amino acid substitutions in a lipase from *Rhizopus delemar*. Thus, R. D. Joerger et al., Lipids, 29 (6), 377-384 (1994) indicates that the variants F95D, F112W and V209W have an altered preference to $C_4$ and $C_8$ acids. R. R. Klein et al., JAOCS, 74 (11), 1401-1407 (1997) shows that the variant V206T+F95D has a higher selectivity for $C_8$ acid. R. R. Klein et al., Lipids, 32 (2), 123-130 (1997) indicates that the variants V209W+F112W, V94W and F95D+F214R have a higher hydrolytic activity towards $C_4$ and $C_8$ acids, and suggests that structural determinants for medium-chain length specificity may reside in the distal end of the acyl binding groove.

## SUMMARY OF THE INVENTION

The inventors have found that the substrate specificity of a lipolytic enzyme can be modified by making alterations to the amino acid sequence in a defined region of the lipolytic enzyme, so as to increase the level of a desired activity or to decrease the level of an undesired activity. Thus, the inventors have developed lipolytic enzymes with a modified amino acid sequence (hereinafter called lipolytic enzyme variants, or variants for short) with a substrate specificity which can be tailored for specific uses.

Accordingly,the invention provides a method of producing a lipolytic enzyme variant and lipolytic enzyme variants prepared by the method. The method comprises:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme,

c) selecting at least one amino acid residue in a region near the active site, near the C-terminal or in the lid region of the parent lipolytic enzyme as described below,

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the resulting variant,

g) testing the activity of the variant on the ester bond in the substrate, and

h) selecting a variant having an altered activity on the ester bond.

Thus, in one aspect, the parent lipolytic enzyme has an alcohol binding site having a glycerol part with an sn2 position, and the amino acid alteration is within 10 Å of the C atom at the sn2 posi- tion of the glycerol part of a substrate triglyceride.

In another aspect, the parent lipolytic enzyme has a structure comprising a catalytic triad consisting of an active Ser, an active Asp and an active His residue, and the amino acid to be altered is either located between the active His residue of the catalytic residue and the C-terminal, or belongs to a set E defined by the following steps:

i) aligning the structure of the lipolytic enzyme with *Rhizomucor miehei* lipase structure 4TGL comprising a catalytic triad and an inhibitor phosphorus atom (4TGL-inhP), so as to minimize the sum of squares of deviation between atoms of the catalytic triads of the two structures,

ii) defining a set A consisting of atoms of the lipolytic enzyme inside a sphere of radius 18 Å with center at 4TGL-inhP,

iii) forming a first plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of the active Asp residue of the parent lipolytic enzyme and defining a set B as a subset of set A consisting of atoms on the same side of the first plane as the Cα atom of the active His residue of the parent lipolytic enzyme,

iv) forming a second plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of the active His residue of the parent lipolytic enzyme and defining a set C as a subset of set A consisting of atoms on the opposite side of the second plane from the Cα atom of the active Asp residue of the parent lipolytic enzyme,

v) forming a set D consisting of atoms belonging to the union of sets B and C, and having a solvent accessibility of 15 or higher, and

vi) forming set E consisting of amino acid residues in the structure which comprise an atom belonging to set D or an atom belonging to the union of sets B and C and located less than 3.5 Å from an atom belonging to set D,

In a third aspect, the lipolytic enzyme has an active site comprising an active His residue, and the alteration is made in the amino acid sequence between the active His residue and the C-terminal.

In yet another aspect of the invention, the amino acid alteration is made among the 10 amino acid residues at the C-terminal.

In a further aspect, the parent lipolytic enzyme has a lid, and the alteration is made in the lid.

The invention also provides a DNA sequence encoding the variant, an expression vector comprising the DNA sequence, a transformed host cell harboring the DNA sequence or the expression vector, and to a method of producing the variant by cultivating the transformed host cell so as to produce the variant and recovering the variant from the resulting broth. Further, the invention provides uses of the variants.

The inventors have also found that a lipolytic enzyme which has lipase and phospholipase activity as well as activity on digalactosyl diglyceride is particularly effective for use in baking, and they designed a screening method for lipolytic enzymes by testing for these activities.

## BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 shows an alignment of lipase sequences.

## DETAILED DESCRIPTION OF THE INVENTION

### Altered activity on selected ester bond in substrate

Compared to the parent lipolytic enzyme, the invention aims to alter the activity on at least one selected ester bond in at least one substrate, i.e. to increase a desired activity, decrease an undesired activity or to change the substrate specificity by decreasing the ratio of an undesired activity to a desired activity.

Thus, an enzyme with increased phospholipase activity may be useful, e.g., in baking or in purification of vegetable oil. It may be desired to increase the hydrolytic activity on digalactosyl-diglyceride (DGDG) for use in baking.

It may be desired to increase the lipase activity for any industrial use where lipases are used. For use in detergents or baking it may be desired to increase the activity on long-chain ($C_{16}$-$C_{20}$) triglycerides, and it may be desired to increase the specificity for long-chain fatty acids by decreasing the ratio of activity on short-chain or medium-chain ($C_4$-$C_8$) fatty acids to the activity on long-chain fatty acids.

For use in, or for use in flavor development in food products (such as cheese ripening) it may be desired to increase the lipase activity on short-chain or medium-chain ($C_4$-$C_8$) triglycerides.

For use as a phospholipase in purification of vegetable oil, it may be desired to decrease the ratio of lipase activity on long-chain ($C_{16}$-$C_{20}$) triglycerides to the phospholipase activity.

**Parent lipolytic Enzyme**

The lipolytic enzyme to be used in the present invention is one that can hydrolyze ester bonds. Such enzymes include, for example, lipases, such as triacylglycerol lipase (EC 3.1.1.3), lipoprotein lipase (EC 3.1.1.34), monoglyceride lipase (EC 3.1.1.23), lysophospholipase, ferulic acid esterase and esterase (EC 3.1.1.1, EC 3.1.1.2). The numbers in parentheses are the systematic numbers assigned by the Enzyme Commission of the International Union of Biochemistry in accordance with the type of the enzymatic reactivity of the enzyme.

The parent lipolytic enzyme may be prokaryotic, particularly a bacterial enzyme, e.g. from *Pseudomonas*. Examples are *Pseudomonas* lipases, e.g. from *P. cepacia* (US 5,290,694, pdb file 1OIL), *P. glumae* (N Frenken et al. (1992), Appl. Envir. Microbiol. 58 3787-3791, pdb files 1TAH and 1QGE), *P. pseudoalcaligenes* (EP 334 462) and *Pseudomonas sp.* strain SD 705 (FERM BP-4772) (WO 95/06720, EP 721 981, WO 96/27002, EP 812 910). The *P. glumae* lipase sequence is identical to the amino acid sequence of *Chromobacterium viscosum* (DE 3908131 A1). Other examples are bacterial cutinases, e.g. from *Pseudomonas* such as *P. mendocina* (US 5,389,536) or *P. putida* (WO 88/09367).

Alternatively, the parent lipolytic enzyme may be eukaryotic, e.g. a fungal lipolytic enzyme such as lipolytic enzymes of the *Humicola* family and the *Zygomycetes* family and fungal cutinases.

Examples of fungal cutinases are the cutinases of *Fusarium solani pisi* (S. Longhi et al., Journal of Molecular Biology, 268 (4), 779-799 (1997)) and *Humicola insolens* (US 5,827,719).

The *Humicola* family of lipolytic enzymes consists of the lipase from *H. lanuginosa* strain DSM 4109 and lipases having more than 50 % homology with said lipase. The lipase from *H. lanuginosa* (synonym *Thermomyces lanuginosus*) is described in EP 258 068 and EP 305 216, and has the amino acid sequence shown in positions 1-269 of SEQ ID NO: 2 of US 5,869,438.

The *Humicola* family also includes the following lipolytic enzymes: lipase from *Penicillium camembertii* (P25234), lipase/phospholipase from *Fusarium oxysporum* (EP 130064, WO 98/26057), lipase from *F. heterosporum* (R87979), lysophospholipase from *Aspergillus foetidus* (W33009), phospholipase A1 from *A. oryzae* (JP-A 10-155493), lipase from *A. oryzae* (D85895), lipase/ferulic acid es-

terase from *A. niger* (Y09330), li- pase/ferulic acid esterase from *A. tubingensis* (Y09331), lipase from *A. tubingensis* (WO 98/45453), lysophospholipase from *A. niger* (WO 98/31790), lipase from *F. solanii* having an isoelectric point of 6.9 and an apparent molecular weight of 30 kDa (WO 96/18729).

The *Zygomycetes* family comprises lipases having at least 50 % homology with the lipase of *Rhizomucor miehei* (P19515). This family also includes the lipases from *Absidia reflexa*, *A. sporophora*, *A. corymbifera*, *A. blakesleeana*, *A. griseola* (all described in WO 96/13578 and WO 97/27276) and *Rhizopus oryzae* (P21811). Numbers in parentheses indicate publication or accession to the EMBL, GenBank, GeneSeqp or Swiss-Prot databases.

It is of particular interest to derive a variant with phospholipase activity from a parent lipolytic enzyme having no or very little phospholipase activity, e.g. corresponding to a ratio of phospholipase activity to lipase activity below 0.1 PHLU/LU or below 50 PHLU/mg.

**Alteration near alcohol binding site**

As already stated, the amino acid sequence of the parent lipolytic enzyme may be modified at a position which near the glycerol part of a substrate triglyceride. This region will be referred to as the "alcohol binding site" of the lipase; it is described in Brzozowski A M et al., Nature, 351: 491 (1991); Uppenberg et al., Biochemistry, 1995, 34, 16838-16851; A. Svendsen, Inform, 5(5), 619-623 (1994).

For the *Rhizomucor miehei* lipase, the extent of the alcohol binding site can be found from the PDB file "5tgl.pdb" available in Structural Classification of Proteins (SCOP) on the Internet, at http://www.rcsb.org/pdb/, showing the complex with the inhibitor n-hexylphosphonate ethyl ester which mimics the substrate. It is described in Derewenda et al. (supra), Brzozowski et al. (supra) and Brady et al. (supra). The sn2 position of this model is the atom CE2.

The variant typically contains no more than 10 alterations in the alcohol binding site, e.g. 1, 2, 3, 4, 5 or 6 alterations.

The alteration may particularly be in that part of the alcohol binding site which comes within 20 positions (e.g. within 10 positions) of the C-terminal.

As already stated, the amino acid sequence of the parent lipolytic enzyme may be modified at a position which is within 10 Å (e.g. within 8 Å, particularly within 6 Å) of the C atom at the sn2 position of the glycerol part of a substrate triglyceride. The following amino acid positions lie within 10 Å of the sn2 position in the *Rhizomucor miehei* lipase: 25, 28, 80-84, 88, 143-146, 175, 203, 205, 254-255, 257-259, 264-

267. The following are within 8 Å: 81-83, 144, 257-258, 265-267, and the following within 6 Å: 82, 144, 257, 266.

In the *Humicola lanuginosa* lipase, the following positions are within 10 Å of the sn2 position: 18, 21, 81-85, 89, 145-148, 172, 201, 203, 255-256, 258-260, 264-267. The following are within 8 Å: 82-84, 89, 146, 258-259, 265-267, and the following within 6 Å: 83, 146, 258, 266.

**Alteration near catalytic triad**

As already stated, in one aspect the parent lipolytic enzyme has a structure comprising a catalytic triad consisting of an active Ser, an active Asp and an active His residue, and the amino acid to be altered belongs to a set defined by a certain procedure described above. The structure may be an open or a closed structure, and it may or may not include a substrate or an inhibitor.

The procedure is conveniently performed by use of software such as MSI's Insight II. It involves alignment with 4TGL, a crystal structure of the lipase from *Rhizomucor miehei* inhibited irreversibly by diethyl p-nitrophenyl phosphate. This is available in Structural Classification of Proteins (SCOP) on the Internet, at http://www.rcsb.org/pdb/, and is described in Derewenda et al. (supra). The *Rhizomucor miehei* lipase comprises a catalytic triad consisting of the amino acid residues S144, D203 and H 257.

For the *Humicola lanuginosa* lipase, the structure 1tib may be used; it is available in Structural Classification of Proteins (SCOP) on the Internet. Using this structure, the set defined by the procedure includes the following positions: 10-23, 26, 40, 55-64, 80-87, 116-117, 119, 145-149, 151, 168, 170, 194, 196-201, 220-222, 224-227, and 254-269.

**Alteration between at C-terminal side of the active His residue**

As stated above, one or more alterations may be made in the amino acid sequence between an active His residue and the terminal, specifically among the 12 amino acids at the C-terminal side of the active His.

The *Humicola lanuginosa* lipase has an active His at H258 and the C-terminal at L269, so this region includes positions 259-269. The *P. cepacia* lipase has an active H286 and the C-terminal at residue 297, so the region includes residues 287-297.

**Alteration near C-terminal**

As stated above, one or more alterations may be made within 10 amino acid positions from the C-terminal of the mature protein,

or at positions corresponding to such positions in the *H. lanuginosa* lipase, i.e. positions 260-269 of the *H. lanuginosa* lipase. Corresponding positions may be found by alignment of the two sequences as described later in this specification.

The lipolytic enzyme variant may be truncated by deleting amino acid residues corresponding to the first 1, 2, 3, 4, 5 or 6 positions at the C-terminal. A truncated variant may have improved thermostability.

Alternatively, the variant may carry a peptide extension at the C-terminal and/or the N-terminal. The C-terminal extension may consist of 1-10 amino acid residues, e.g. A, P, AG, DG, PG, AGG, PVGF, AGRF, PRGF, AGGF or AGGFS; or it may consist of 40-50 residues, e.g., consisting of the 48 C-terminal residues of the *Fusarium oxysporum* lipase AGGFSWRRYRSAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS. The C-terminal extension may increase the phospholipase activity.

Some alterations in the region overlapping with the alcohol binding site are described below.

A specific alteration is a substitution at a position corresponding to G266 in the *Humicola lanuginosa* lipase, specifically with an amino acid of intermediate size, e.g. A, C, D, N, L, I, S, T, P or V. Such alteration alone has been found sufficient to increase the phospholipase activity.

Other specific alterations are such that alter the tertiary structure, e.g. by introducing bulky side chains or by disrupting the bond angles, e.g. by introducing Pro. Such alterations may be made at positions corresponding to positions G263, L264, I265, T267 or L269 in the *Humicola lanuginosa* lipase. Some specific substitutions are G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; T267A,Q or L269N.

**Alteration in lid**

As stated above, the amino acid sequence of the prent lipolytic enzyme may be modified in the lid region of the parent lipolytic enzyme. This region is described in Brady et al., Nature 343, 1990, pp. 767-770 and in Brzozowski A M et al., Nature, 351: 491 (1991). In the *H. lanuginosa* lipase, the lid is located at positions 80-100, and the modification may particularly be made at positions 82-98, e.g. 91-98.

The variant typically contains no more than 5 alterations in the lid region; it may contain 0, 1, 2 or 3 alterations. A specific alteration is a substitution of an amino acid corresponding to G91, L93, N94, D96, K98, L97 and/or E99 in the *Humicola lanu-*

*ginosa* lipase with a neutral or positively charged amino acid, e.g. a substitution corresponding to G91A,T, L93K, N94D, D96S,W,G, L97Q, K98D,F,E and/or E99K,D.

Specifically, a variant with an alteration in the lid region also contains one or more alterations near the catalytic triad, near the substrate binding site or near the C-terminal.

**Lipolytic enzyme variants**

The lipolytic enzyme variant of the invention comprises one or more alterations of an amino acid residue in any of the regions described above. Each alteration may be a deletion or a substitution of the amino acid residue, or it may be an insertion before or after the amino acid residue. If the amino acid residue is at the C-terminal, the insertion may be a C-terminal extension. An insertion typically consists of 1-5 amino acid residues, e.g. 1-2, and a C-terminal extension may consist of 1-50 or 2-10 amino acid residues.

The total number of alterations in the above regions is typically not more than 20, e.g. not more than 10 or not more than 5, and there may be as little as 1 or 2 alterations in the above regions.

In addition, the lipolytic enzyme variant of the invention may optionally include other modifications of the parent enzyme, typically not more than 10, e.g. not more than 5 such modifications.

The variant generally has a homology with the parent lipolytic enzyme of at least 80 %, e.g. at least 85 %, typically at least 90 % or at least 95 %.

The variant of the invention may further comprise a peptide extension at the N-terminal, e.g. consisting of 1-15 (particularly 4-10) amino acid residues, and specifically comprising 1, 2 or 3 positively charged amino acids. Some specific N-terminal peptide extensions are AS, SPIRR, E1RP, E1SPIRPRP, E1SPPRRP and E1SPIRPRP. Further, any peptide extension described in WO 97/04079 and WO 97/07202 may be used.

**Specific variants**

To prepare variants of a lipolytic enzyme of the *Humicola* family, the amino acid alterations may specifically be made at positions corresponding to 20-25, 56-64, 81-85 or 255-269 in the *Humicola lanuginosa* lipase. Thus, the alteration may be a substitution, deletion or insertion at a position corresponding to A20, Y21, G23, K24, N25, V63, R81, G82, R84, A257, W260, Y261, F262 or G266 (e.g. excluding G23C, K24C, R81C), a substitution of an amino acid corresponding to C268 or L269.

Some specific alterations are substitutions corresponding to the following in *H.* lanuginosa lipase: Y21V/I/L/A/G/M/W/P/F/N/Q/S/T, V60V/I/L/A/G/M/W/P/F/N/Q/S/T, G61V/I/L/A/G/M/W/P/F/N/Q/S/T, D62E/A/V, S83T, R84K/L/W, P256A, G263E,Q,R,F, L264A,C,P,F,G,I, I265L,N,F G266D/E or T267A,Q,P,S,E, or an insertion corresponding to T267GS or T267GL.

To alter the activity towards short-chain ($C_4$-$C_8$) fatty acids in triglycerides, alterations may be made at positions corresponding to Y21, E56, D57, V60, G61, D62, R81, S83, R84, L259, Y261 or G266, e.g. a substitution corresponding to Y21V/I, V60G, D62E/A/V, S83T, R84K/L/W or G266D/E.

To increase the activity for DGDG, alterations may be made at positions corresponding to Y21, G23, N26, D57, D62, R81, S83, R84, S85, G266, T267 or L269; e.g., two or more such alterations may be made, e.g. together with one or more alterations in the lid region. To increase the phospholipase activity, alterations may be made at positions corresponding to R81, R84, S85, or 263-267, e.g. G266 or T267.

To prepare variants of a *Pseudomonas* lipase, amino acid modifications may be made at positions corresponding to 12-13, 16-34, 45-52, 59-66, 68, 86-87, 107-109, 111, 143-153, 155, 157-158, 207-212, 228, 230, 242-249, 264, 279-280, 282-297, 301-302, 304-305, 307-308 in the *P. cepacia* lipase, particularly L17/L17, T18/A18, Y29/Y29, L287/L286, E289/E288, I290/I289, Q292/Q291 or L293/L292 in the *P. cepacia/P. glumae* lipase.

Specific variants of the *H. lanuginosa* lipase are disclosed in the examples. Corresponding alterations may be made in other parent lipolytic enzymes. Further variants may be derived from these by omitting amino acid modifications at positions 1, 106, 186, 225, 232, 237, 239 or 274. Variants with 274S may optionally have a further C-terminal extension of WRRYR-SAESVDKRATMTDAELEKKLNSYVQMDKEYVKNNQARS (corresponding to the C-terminal of the *F. oxysporum* lipase) in full or truncated form.

**Nomenclature for amino acid alterations**

The nomenclature used herein for defining mutations is basically as described in WO 92/05249. Thus, G91A indicates substitution of G in position 91 with A. T267A,Q indicates substitution of T at position 267 with A or Q. E1E,D,A indicates that E1 is unchanged or is substituted with D or A.

T267stop indicates a stop codon, i.e. deletion of T267 and all following amino acids (i.e. C268 and L269). 270P, 271V indicates a C-terminal extension of PV (i.e. at new positions 270 and 271). -G266 indicates deletion of G at position 266. Parentheses indicate that the alteration is optional, or in examples that the alteration

is uncertain. SPIRR indicates an N- terminal extension. D266 may refer to the position or to substitution with any amino acid (except D).

E1SPPCGRRP or SPPCGRRP(- E) indicates a substitution of E1 with SPPCGRRP, i.e. a peptide addition at the N-terminal. T267GS indicates a substitution of T267 with GS, or in other words the substitution T267G and an insertion of S between G267 and C268.

**Homology and alignment**

For purposes of the present invention, the degree of homology may be suitably determined by means of computer programs known in the art, such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45), using GAP with the following settings for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

In the present invention, corresponding (or homologous) positions in the lipase sequences of *Rhizomucor miehei* (rhimi), *Rhizopus delemar* (rhidl), *Thermomyces lanuginosa* (former; *Humicola lanuginosa*) (SP400), *Penicillium camembertii* (Pcl) and *Fusarium oxysporum* (FoLnp11), are defined by the alignment shown in Figure 1.

To find the homologous positions in lipase sequences not shown in the alignment, the sequence of interest is aligned to the sequences shown in Figure 1. The new sequence is aligned to the present alignment in Fig. 1 by using the GAP alignment to the most homologous sequence found by the GAP program. GAP is provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-45). The following settings are used for polypeptide sequence comparison: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

**Variants with phospholipase activity**

As described above, the variant of the invention may have a higher phospholipase activity than the parent lipolytic enzyme. By the monolayer method described later in this specification, the variant may have a phospholipase activity of at least 0.1 nmol/min at pH 5.

By the PHLU method described later in this specification, the variant may have a phospholipase activity of at least 100 PHLU/mg (mg of pure enzyme protein), particularly at least 500 PHLU/mg. The variant has a ratio of phospholipase activity to lipase activity (both measured at pH 7) of at least 0.1 PHLU/LU, e.g. at least 0.5, particularly at least 2.

The variants of the invention may have the ability to hydrolyze intact phospholipid, as demonstrated by the PHLU method. They may have $A_1$ and/or $A_2$ activity, so they may be able to hydrolyze one or both fatty acyl groups in the phospholipid.

**pH optimum**

Many variants of the *Humicola lanuginosa* lipase have an alkaline pH optimum for lipase activity and an acid pH optimum for phospholipase activity (e.g. pH 9-10 for lipase and pH 4-6 for phospholipase). Such variants can be used at acid pH (e.g. in oil degumming, described later), as phospholipases with very low concomitant lipase activity.

However, some variants of the *Humicola lanuginosa* lipase which include the substitution G266D,E have pH optima for both lipase and phospholipase activities around pH 5-6. Such variants may be used at acid pH when both lipase and phospholipase activities are desired, e.g. in baking.

**Thermostability**

The thermostability of the variant can conveniently be evaluated by means of Differential Scanning Calorimetry (DSC). Depending on exact mutations, the variants of the invention generally have similar or slightly lower thermostability than the parent lipolytic enzyme.

The temperature at the top of the denaturation peak ($T_d$) of the lipase from *Humicola lanuginosa* when heated at 90 deg/hr at pH 5 is just above 70 °C (=$T_d$). $T_d$ for the variants of the invention is generally 5-10 degrees lower

**Use of variant**

Depending on the substrate specificity, variants of the invention can be used, e.g., in filtration improvement, vegetable oil treatment, baking, detergents, or preparation of lysophospholipid.

Improvement of filtration

A variant with lysophospholipase activity can be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the variant. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Vegetable oil treatment

A variant with phospholipase activity can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the variant so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g. vegetable oil such as soy bean oil, rape seed oil and sunflower oil. The treatment may be carried out at acid pH, e.g. pH 3-5. Advantageously, a variant can be selected so as to have a high phospholipase activity and a low lipase activity at low pH, due to different pH optima of the two activities.

The process for oil treatment can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahlke & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

Miscellaneous uses of phospholipase

A variant with phospholipase activity can be used to prepare lysophospholipid (e.g. lyso-lecithin) by treating the corresponding phospholipid with the variant, e.g. as described in EP 870840, JP-A 10-42884, JP-A 4-135456 or JP-A 2-49593. The variant can also be used to make mayonnaise, e.g. as described in EP 628256, EP 398666 or EP 319064.

A variant with phospholipase activity may also be used in the processing of dairy and other food products, e.g. as described in EP 567,662 (Nestlé), EP 426,211 (Unilever), EP 166,284 (Nestlé), JP-A 57-189638 (Yakult) or US 4,119,564 (Unilever).

The variant may be used leather treatment, as described in JP-A 7-177884 (Kao).

Baking

A variant with phospholipase and/or DGDGase activity can be used in the preparation of dough, bread and cakes, e.g. to increase dough stability and dough handling properties, or to improve the elasticity of the bread or cake. Thus, the variant can be used in a process for making bread, comprising adding the variant to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.), EP 426211 (Unilever) or WO 99/53769 (Novo Nordisk).

It is particularly advantageous to use the variant together with an anti-staling endo-amylase and optionally also to add a phospholipid, to reduce-staling of the bread and particularly to improve softness of the bread in the first 24 hours after baking. The endo-amylase may be a maltogenic α-amylase (e.g. from *Bacillus sp.*, such as Novamyl® from Novo Nordisk) or a fungal or bacterial α-amylase, e.g. from *Aspergillus* or *Bacillus*, particularly *A. oryzae*, *B. licheniformis* or *B. amyloliquefaciens*.

In baking, the variant may have a low activity on short-chain or medium-chain ($C_4$-$C_8$), e.g. corresponding to a SLU/LU ratio above 3. The use of such a variant may avoid or suppress the development of an undesired flavor due to the release of short-chain fatty acids. The variant may have activity on triglycerides and phospholipid as well as DGDG.

Cheese flavor

A variant with activity towards short-chain fatty acyl groups may be used to release free fatty acids (FFA) for flavor development in food products, e.g. in cheese ripening, e.g. as described in M. Hanson, ZFL, 41 (10), 664-666 (1990)).

Lipolytic enzyme variants with increased release of short chain fatty compared to long chain fatty acids from milk fat are useful in cheese production, e.g. for flavor enhancement or shortening of the ripening times for ripened cheeses, like cheddar or parmesan. Another application for such lipolytic enzyme variants is for enzyme modified cheese (EMC) for use as flavoring for various food products including process cheese, dressing and snack.

Release of short chain fatty acids, like butyric acid, is essential for the development of cheese flavor, whereas release of long chain fatty acids, like oleic acid, give rise to off flavors. Lipolytic enzyme variants for cheese applications, including EMC, should have SLU/LU ratio of less than 0.5, e.g. less than 0.25, most preferable less than 0.1

Use in detergent

The variant may be used as a detergent additive, e.g. at a concentration (expressed as pure enzyme protein) of 0.001-10 (e.g. 0.01-1) mg per gram of detergent or 0.001-100 (e.g. 0.01-10) mg per liter of wash liquor.

In detergents, the variant may have a high activity on long-chain triglycerides ($C_{16}$-$C_{20}$) to improve the removal of fatty soiling. The variant may have phospholipase activity. The variant may have low activity towards short-chain ($C_4$-$C_8$) fatty acids in triglycerides, e.g. corresponding to a SLU/LU ratio above 10. The use of such a variant may avoid or suppress the development of an undesired odor due to the release of short-chain fatty acids.

Variants having both lipase and phospholipase activity at alkaline pH may be used in detergents.

**Detergent composition**

The detergent composition of the invention may for example be formulated as a hand or machine laundry detergent composition including a laundry additive composition suitable for pre-treatment of stained fabrics and a rinse added fabric softener composition, or be formulated as a detergent composition for use in general household hard surface cleaning operations. In a laundry detergent, the variant may be effective for the removal of fatty stains, for whiteness maintenance and for dingy cleanup. A laundry detergent composition may be formulated as described in WO 97/04079, WO 97/07202, WO 97/41212, PCT/DK WO 98/08939 and WO 97/43375.

The detergent composition of the invention may particularly be formulated for hand or machine dishwashing operations. e.g. as described in GB 2,247,025 (Unilever) or WO 99/01531 (Procter & Gamble). In a dishwashing composition, the variant may be effective for removal of greasy/oily stains, for prevention of the staining /discoloration of the dishware and plastic components of the dishwasher by highly colored components and the avoidance of lime soap deposits on the dishware.

The detergent composition of the invention may be in any convenient form, e.g., a bar, a tablet, a powder, a granule, a paste or a liquid. A liquid detergent may be aqueous, typically containing up to 70 % water and 0-30 % organic solvent, or non-aqueous.

The detergent composition comprises one or more surfactants, which may be non-ionic including semi-polar and/or anionic and/or cationic and/or zwitterionic. The surfactants are typically present at a level of from 0.1% to 60% by weight, e.g. 0.5-40 %, such as 1-30 %, typically 1.5-20 %.

When included therein the detergent will usually contain from about 1% to about 40% of an anionic surfactant such as linear alkylbenzenesulfonate, alpha-olefinsulfonate, alkyl sulfate (fatty alcohol sulfate), alcohol ethoxysulfate, secondary alkanesulfonate, alpha-sulfo fatty acid methyl ester, alkyl- or alkenylsuccinic acid or soap.

When included therein the detergent will usually contain from about 0.2% to about 40% of a non-ionic surfactant such as alcohol ethoxylate, nonyl-phenol ethoxylate, alkylpolyglycoside, alkyldimethylamine-oxide, ethoxylated fatty acid monoethanol-amide, fatty acid monoethanolamide, polyhydroxy alkyl fatty acid amide, or N-acyl N-alkyl derivatives of glucosamine ("glucamides").

The invention also provides a detergent additive comprising the variant of the invention. The detergent additive as well as the detergent composition may comprise one or more other enzymes such as a protease, a lipase, a cutinase, an amylase, a carbohydrase, a cellulase, a pectinase, a mannanase, an arabinase, a galactanase, a xylanase, an oxidase, e.g., a laccase, and/or a peroxidase.

In general the properties of the chosen enzyme(s) should be compatible with the selected detergent, (i.e. pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

Proteases: Suitable proteases include those of animal, vegetable or microbial origin. Microbial origin is preferred. Chemically modified or protein engineered mutants are included. The protease may be a serine protease or a metallo protease, e.g. an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from Bacillus, e.g., subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (described in WO 89/06279). Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270 and WO 94/25583.

Examples of useful proteases are the variants described in WO 92/19729, WO 98/20115, WO 98/20116, and WO 98/34946, especially the variants with substitutions in one or more of the following positions: 27, 36, 57, 76, 87, 97, 101, 104, 120, 123, 167, 170, 194, 206, 218, 222, 224, 235 and 274.

Specific commercially available protease enzymes include Alcalase ®, Savinase ®, Primase ®, Duralase ®, Esperase ®, and Kannase ® (Novo Nordisk A/S), Maxatase ®, Maxacal ®, Maxapem ®, Properase ®, Purafect ®, Purafect OxP ®, FN2 ™, and FN3 ™ (Genencor International Inc.).

Cellulases: Suitable cellulases include those of bacterial or fungal origin. Chemically modified or protein engineered mutants are included. Suitable cellulases

include cellulases from the genera *Bacillus, Pseudomonas, Humicola, Fusarium, Thielavia, Acremonium*, e.g. the fungal cellulases produced from *Humicola insolens, Myceliophthora thermophila* and *Fusarium oxysporum* disclosed in US 4,435,307, US 5,648,263, US 5,691,178, US 5,776,757 and WO 89/09259.

Especially suitable cellulases are the alkaline or neutral cellulases having colour care benefits. Examples of such cellulases are cellulases described in EP 0 495 257, EP 0 531 372, WO 96/11262, WO 96/29397, WO 98/08940. Other examples are cellulase variants such as those described in WO 94/07998, EP 0 531 315, US 5,457,046, US 5,686,593, US 5,763,254, WO 95/24471, WO 98/12307 and PCT/DK98/00299.

Commercially available cellulases include Celluzyme ®, and Carezyme ® (Novo Nordisk A/S), Clazinase ®, and Puradax HA ® (Genencor International Inc.), and KAC-500(B) ® (Kao Corporation).

Peroxidases/Oxidases: Suitable per-oxidases/oxidases include those of plant, bac-terial or fungal origin. Chemically modified or protein engineered mutants are included. Examples of useful peroxidases include peroxidases from *Coprinus*, e.g. from *C. cinereus*, and variants thereof as those described in WO 93/24618, WO 95/10602, and WO 98/15257.

Commercially available peroxidases include Guardzyme ® (Novo Nordisk A/S).

The detergent enzyme(s) may be included in a detergent composition by adding separate additives containing one or more enzymes, or by adding a combined additive comprising all of these enzymes. A detergent additive of the invention, i.e. a separate additive or a combined additive, can be formulated e.g. as a granulate, a liquid, a slurry, etc. Specific detergent additive formulations are granulates, in particular non-dusting granulates, liquids, in particular stabilized liquids, or slurries.

Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethyleneglycol, PEG) with mean molar weights of 1000 to 20000; ethoxylated nonyl-phenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in GB 1483591. Liquid enzyme preparations may, for in-stance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lac-

tic acid or boric acid according to established methods. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent may contain 0-65 % of a detergent builder or complexing agent such as zeolite, diphosphate, tripho-sphate, phosphonate, carbonate, citrate, nitrilotriacetic acid, ethylenediaminetetraacetic acid, diethylenetri-aminepen-taacetic acid, alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst).

The detergent may comprise one or more polymers. Examples are carboxymethylcellulose, poly(vinyl-pyrrolidone), poly (ethylene glycol), poly(vinyl alcohol), poly(vinylpyridine-N-oxide), poly(vinylimidazole), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H2O2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine or nonanoyloxybenzenesul-fonate. Alternatively, the bleaching system may comprise peroxyacids of e.g. the amide, imide, or sulfone type.

The enzyme(s) of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g., a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative, e.g., an aromatic borate ester, or a phenyl boronic acid derivative such as 4-formylphenyl boronic acid, and the composition may be formulated as described in e.g. WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as e.g. fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil redeposition agents, dyes, bactericides, optical brighteners, hydrotropes, tarnish inhibitors, or perfumes.

It is at present contemplated that in the detergent compositions any enzyme, in particular the variant of the invention, may be added in an amount corresponding to 0.01-100 mg of enzyme protein per liter of wash liquor, e.g. 0.05-5 mg of enzyme protein per liter of wash liquor, in particular 0.1-1 mg of enzyme protein per liter of wash liquor.

The variant of the invention may additionally be incorporated in the detergent formulations disclosed in WO 97/07202 which is hereby incorporated as reference.

**Methods for preparing enzyme variants**

The enzyme variant of the invention can be prepared by methods known in the art, e.g. as described in WO 97/04079 (Novo Nordisk). The following describes methods for the cloning of enzyme-encoding DNA sequences, followed by methods for generating mutations at specific sites within the enzyme-encoding sequence.

**Cloning a DNA sequence encoding a enzyme**

The DNA sequence encoding a parent enzyme may be isolated from any cell or microorganism producing the enzyme in question, using various methods well known in the art. First, a genomic DNA and/or cDNA library should be constructed using chromosomal DNA or messenger RNA from the organism that produces the enzyme to be studied. Then, if the amino acid sequence of the enzyme is known, labeled oligonucleotide probes may be synthesized and used to identify enzyme-encoding clones from a genomic library prepared from the organism in question. Alternatively, a labeled oligonucleotide probe containing sequences homologous to another known enzyme gene could be used as a probe to identify enzyme-encoding clones, using hybridization and washing conditions of lower stringency.

Yet another method for identifying enzyme-encoding clones would involve inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing a substrate for enzyme (*i.e.* maltose), thereby allowing clones expressing the enzyme to be identified.

Alternatively, the DNA sequence encoding the enzyme may be prepared synthetically by established standard methods, *e.g.* the phosphoroamidite method described S.L. Beaucage and M.H. Caruthers, (1981), Tetrahedron Letters 22, p. 1859-1869, or the method described by Matthes et al., (1984), EMBO J. 3, p. 801-805. In the phosphoroamidite method, oligonucleotides are synthesized, *e.g.* in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate, the fragments corresponding to various parts of the entire DNA sequence), in accordance with standard techniques. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., (1988), Science 239, 1988, pp. 487-491.

**Site-directed mutagenesis**

Once a enzyme-encoding DNA sequence has been isolated, and desirable sites for mutation identified, muta- tions may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. In a specific method, a single-stranded gap of DNA, the enzyme-encoding sequence, is created in a vector carrying the enzyme gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in with DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984), Biotechnology 2, p. 646-639. US 4,760,025 discloses the introduction of oligonucleotides encoding multiple mutations by performing minor alterations of the cassette. However, an even greater variety of mutations can be introduced at any one time by the Morinaga method, because a multitude of oligonucleotides, of various lengths, can be introduced.

Another method for introducing mutations into enzyme-encoding DNA sequences is described in Nelson and Long, (1989), Analytical Biochemistry 180, p. 147-151. It involves the 3-step generation of a PCR fragment containing the desired mutation introduced by using a chemically synthesized DNA strand as one of the primers in the PCR reactions. From the PCR-generated fragment, a DNA fragment carrying the mutation may be isolated by cleavage with restriction endonucleases and reinserted into an expression plasmid.

Further, Sierks. et al., (1989) "Site-directed mutagenesis at the active site Trp120 of *Aspergillus awamori* glucoamylase. Protein Eng., 2, 621-625; Sierks et al., (1990), "Catalytic mechanism of fungal glucoamylase as defined by mutagenesis of Asp176, Glu179 and Glu180 in the enzyme from *Aspergillus awamori*". Protein Eng. vol. 3, 193-198; also describes site-directed mutagenesis in an *Aspergillus* glucoamylase.

**Expression of enzyme variants**

According to the invention, a DNA sequence encoding the variant produced by methods described above, or by any alternative methods known in the art, can be expressed, in enzyme form, using an expression vector which typically includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes.

**Expression vector**

The recombinant expression vector carrying the DNA sequence encoding a enzyme variant of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. The vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. Examples of suitable expression vectors include pMT838.

**Promoter**

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA sequence encoding a enzyme variant of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dag*A promoters, the promoters of the *Bacillus licheniformis* α-amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α-amylase (*amyQ*), the promoters of the *Bacillus subtilis* xylA and xylB genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, the TPI (triose phosphate isomerase) promoter from *S. cerevisiae* (Alber et al. (1982), J. Mol. Appl. Genet 1, p. 419-434, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

**Expression vector**

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the α-amylase variant of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B. subtilis* or *B. licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Furthermore, the vector may comprise *Aspergillus* selection markers such as amdS, argB, niaD and sC, a marker giving rise to hygromycin resistance, or the selection may be accomplished by co-transformation, *e.g.* as described in WO 91/17243.

The procedures used to ligate the DNA construct of the invention encoding a enzyme variant, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

**Host Cells**

The cell of the invention, either comprising a DNA construct or an expression vector of the invention as defined above, is advantageously used as a host cell in the recombinant production of a enzyme variant of the invention. The cell may be transformed with the DNA construct of the invention encoding the variant, conveniently by integrating the DNA construct (in one or more copies) in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, *e.g.* by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

The cell of the invention may be a cell of a higher organism such as a mammal or an insect, but may be a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are Gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gramnegative bacteria such as *E.coli*. The transformation of the bacteria may, for instance, be effected by protoplast transformation or by using competent cells in a manner known *per se*.

The yeast organism may favorably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, *e.g. Saccharomyces cerevisiae*.

The host cell may also be a filamentous fungus *e.g.* a strain belonging to a species of *Aspergillus*, such as *Aspergillus oryzae* or *Aspergillus niger, or* a strain of *Fusarium*, such as a strain of *Fusarium oxysporium*, *Fusarium graminearum* (in the perfect state named *Gribberella zeae,* previously *Sphaeria zeae, synonym with Gibberella roseum and Gibberella roseum* f. sp. *cerealis)*, or *Fusarium sulphureum (*in the prefect state named *Gibberella puricaris,* synonym with *Fusarium trichothecioides, Fusarium bactridioides, Fusarium sambucium, Fusarium roseum,* and *Fusarium roseum var. graminearum), Fusarium cerealis (*synonym with *Fusarium crokkwellnse), or Fusarium venenatum.*

In a specific embodiment of the invention the host cell is a protease deficient of protease minus strain.

This may for instance be the protease deficient strain *Aspergillus oryzae* JaL 125 having the alkaline protease gene named "alp" deleted. This strain is described in WO 97/35956 (Novo Nordisk).

Filamentous fungi cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of *Aspergillus* as a host micro-organism is described in EP 238 023 (Novo Nordisk A/S), the contents of which are hereby incorporated by reference.

**Method of producing the enzyme variant of the invention**

The enzyme variant of the invention may be produced by a method comprising cultivating a host cell under conditions conducive to the production of the variant and recovering the variant from the cells and/or culture medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the enzyme variant of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (*e.g.* as described in catalogues of the American Type Culture Collection).

The enzyme variant secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures, including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by the use of chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

**Expression of variant in plants**

The present invention also relates to a transgenic plant, plant part or plant cell which has been transformed with a DNA sequence encoding the variant of the invention so as to express and produce this enzyme in recoverable quantities. The enzyme may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the recombinant enzyme may be used as such.

The transgenic plant can be dicotyledonous or monocotyledonous, for short a dicot or a monocot. Examples of monocot plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as festuca, lolium, temperate grass, such as Agrostis, and cereals, e.g. wheat, oats, rye, barley, rice, sorghum and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous (family Brassicaceae), such as cauliflower, oil seed rape and the closely related model organism Arabidopsis thaliana.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers. In the present context, also specific plant tissues, such as chloroplast, apoplast, mitochondria, vacuole, peroxisomes and cytoplasm are considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part.

Also included within the scope of the invention are the progeny of such plants, plant parts and plant cells.

The transgenic plant or plant cell expressing the variant of the invention may be constructed in accordance with methods known in the art. In short the plant or plant cell is constructed by incorporating one or more expression constructs encoding the variant of the invention into the plant host genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

Conveniently, the expression construct is a DNA construct which comprises a gene encoding the variant of the invention in operable association with appropriate regulatory sequences required for expression of the gene in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences is determined, eg on the basis of when, where and how the enzyme is desired to be expressed. For instance, the expression of the gene encoding the variant of the invention may be constitutive or in-

ducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are eg described by Tague et al, Plant, Phys., 86, 506, 1988.

For constitutive expression the 35S-CaMV promoter may be used (Franck et al., 1980. Cell 21: 285-294). Organ-specific promoters may eg be a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990. Annu. Rev. Genet. 24: 275-303), or from metabolic sink tissues such as meristems (Ito et al., 1994. Plant Mol. Biol. 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin or albumin promoter from rice (Wu et al., Plant and Cell Physiology Vol. 39, No. 8 pp. 885-889 (1998)), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* described by Conrad U. et al, Journal of Plant Physiology Vol. 152, No. 6 pp. 708-711 (1998), a promoter from a seed oil body protein (Chen et al., Plant and cell physiology vol. 39, No. 9 pp. 935-941 (1998), the storage protein napA promoter from Brassica napus, or any other seed specific promoter known in the art, eg as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the rbcs promoter from rice or tomato (Kyozuka et al., Plant Physiology Vol. 102, No. 3 pp. 991-1000 (1993), the chlorella virus adenine methyltransferase gene promoter (Mitra, A. and Higgins, DW, Plant Molecular Biology Vol. 26, No. 1 pp. 85-93 (1994), or the aldP gene promoter from rice (Kagaya et al., Molecular and General Genetics Vol. 248, No. 6 pp. 668-674 (1995), or a wound inducible promoter such as the potato pin2 promoter (Xu et al, Plant Molecular Biology Vol. 22, No. 4 pp. 573-588 (1993).

A promoter enhancer element may be used to achieve higher expression of the enzyme in the plant. For instance, the promoter enhancer element may be an intron which is placed between the promoter and the nucleotide sequence encoding the enzyme. For instance, Xu et al. *op cit* disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The DNA construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, micro injection, particle bombardment, biolistic transformation, and electroporation (Gasser et al, Science, 244, 1293; Potrykus, Bio/Techn. 8, 535, 1990; Shimamoto et al, Nature, 338, 274, 1989).

Presently, *Agrobacterium tumefaciens* mediated gene transfer is the method of choice for generating transgenic dicots (for review Hooykas & Schilperoort, 1992.

Plant Mol. Biol. 19: 15-38), however it can also be used for transforming monocots, although other transformation methods are generally used for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992. Plant J. 2: 275-281; Shimamoto, 1994. Curr. Opin. Biotechnol. 5: 158-162; Vasil et al., 1992. Bio/Technology 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh S, et al., Plant Molecular biology Vol. 21, No. 3 pp. 415-428 (1993).

Following transformation, the transformants having incorporated the expression construct are selected and regenerated into whole plants according to methods well-known in the art.

## MATERIALS AND METHODS

### Lipase activity on tributyrin (LU)

A substrate for lipase is prepared by emulsifying tributyrin (glycerin tributyrate) using gum Arabic as emulsifier. The hydrolysis of tributyrin at 30 °C at pH 7 is followed in a pH-stat titration experiment. One unit of lipase activity (1 LU) equals the amount of enzyme capable of releasing 1 µmol butyric acid/min at the standard conditions.

### Lipase activity on triolein (SLU)

The lipolytic activity may be determined using olive oil as substrate.

In this SLU method, the lipase activity is measured at 30 C and pH 9 with a stabilized olive oil emulsion (Sigma catalog No. 800-1) as the substrate, in a 5 mM Tris buffer containing 40 mM NaCl and 5 mM calcium chloride. 2.5 ml of the substrate is mixed with 12.5 ml buffer, the pH is adjusted to 9, 0.5 ml of diluted lipase sample is added, and the amount of oleic acid formed is followed by titration with a pH stat.

One SLU is the amount of lipase which liberates 1 mole of titratable oleic acid per minute under these conditions.

### Phospholipase activity

The following assay methods were used for qualitative or quantitative determination of phospholipase activity.

Phospholipase activity (PHLU)

Phospholipase activity (PHLU) is measured as the release of free fatty acids from lecithin. 50 µl 4% L-alpha- phosphatidylcholine (plant lecithin from Avanti), 4 % Triton X-100, 5 mM $CaCl_2$ in 50 mM HEPES, pH 7 is added 50 µl enzyme solution diluted to an appropriate concentration in 50 mM HEPES, pH 7. The samples are incubated for 10 min at 30 °C and the reaction stopped at 95 °C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids are determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture is added 250 µl Reagent A and incubated 10 min at 37 °C. Then 500 µl Reagent B is added and the sample is incubated again, 10 min at 37 °C. The absorption at 550 nm is measured using an HP 8452A diode array spectrophotometer. Samples are run in at least in duplicates. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95 °C) + substrate) are included. Oleic acid is used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min at these conditions.

Phospholipase activity (LEU)

Lecithin is hydrolyzed under constant pH and temperature, and the phospholipase activity is determined as the rate of titrant (0.1N NaOH) consumption during neutralization of the liberated fatty acid.

The substrate is soy lecithin (L-α-Phosphotidyl-Choline), and the conditions are pH 8.00, 40.0°C, reaction time 2 min. The unit is defined relative to a standard.

Phospholipase monolayer assay

On a thoroughly purified surface of a buffer solution (either 10 mM Glycin, pH 9.0 or 10 mM NaOAc, pH 5.0; 1 mM CaCl2, 25°C) a monolayer of Di-Decanoyl-Phosphatidyl Choline (DDPC) is spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m, corresponding to a mean molecular area of DDPC of approx. 63 $Å^2$/molec. A solution containing approximately 60 µg (micro gram) enzyme is injected through the monolayer into the subphase of the re-action compartment (cylinder with surface area 2230 mm2 and reaction volume 56570 mm3) in the "zero-order trough". Enzymatic activity is manifested through the speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolyzed into more water soluble reaction products. Having verified that the aqueous solubility of the reaction products (capric acid and MDPC) are considerably higher than for DDPC the number of DDPC-molecules hydrolyzed per min-

ute by the enzyme is estimated from the mean molecular area (MMA) of DDPC. The results are calculated on basis of average barrier speed over the first 5 minutes of hydrolysis.

The result is considered positive for phospholipase if the barrier moves at more than 2 mm/min.

Plate assay 1

A) 50 ml 2% agarose in purified water is melted/stirred for 5 minutes and cooled to 60 - 63°C.

B) 50 ml 2% plant L-alpha-Phosphatidylcholine 95% in 0,2M NaOAc, 10 mM $CaCl_2$, pH 5,5 at 60°C in 30 min. is blended in 15 sec. with ultrathorax.

Equal volumes of 2% agarose and 2% Lecithin (A and B) are mixed, and an equal volume of 1 % Triton X-100 is added to this mixture. 250 µl 4 mg/ml crystal violet in purified water is added as indicator. The mixture is poured into appropriate petri dishes (e.g. 30 ml in 14cm Ø dish), and appropriate holes are made in the agar (3-5 mm) for application of enzyme solution.

The enzyme sample is diluted to a concentration corresponding to $OD_{280}$ = 0.5 and 10 microliter is applied into holes in the agarose/lecithin-matrix. Plates are incubated at 30°C and reaction zones in the plates are identified after approx. 4-5 hours and/or after approx. 20 hours incubation. The *Humicola lanuginosa* lipase is used as a control, and the presence of a larger clearing zone than the control is taken as a positive result for phospholipase activity.

In a variation of this assay, the addition of Triton X-100 is omitted.

Plate assay 2

10 g agarose is melted in 550 ml H2O by boiling in a microwave oven. After cooling to 60-70°C the following ingredients are added:

250 ml of a 0.4 M Citrate buffer (pH 4.5 or pH 7.1)

200 ml 3% lecithin (from Avanti) in 2% Triton-X 100

2 ml 2% crystal violet

30 ml of the mixture is poured into 14 cm Ø petri dishes.

The plates are incubated after application of enzyme samples, and the results are interpreted as for Plate assay 1.

**Digalactosyl diglyceride hydrolyzing (DGDGase) activity**

Monolayer assay 1

On a thoroughly purified surface of a buffer solution (10 mM NaOAc, pH 5.5; 1 mM CaCl2, 25°C; 10 mM beta-cyclodextrin (Sigma C-4767)) a monolayer of DGDG (Sigma (D4651)) is spread from a chloroform solution. After relaxation of the monolayer (evaporation of chlorofom) the surface pressure is adjusted to 15 mN/m. A solution containing approximately 60 µg (micro gram) enzyme is injected through the monolayer into the subphase of the re-action compartment (cylinder with surface area 2230 $mm^2$ and reaction volume 56570 $mm^3$) in the "zero-order trough". Enzymatic activity is manifested through increased speed of a mobile barrier compressing the monolayer in order to maintain constant surface pressure as insoluble substrate molecules are hydrolyzed into more water soluble reaction products (in presence of beta cyclodextrin).

The result is considered positive for DGDGase if the barrier moves at more than 1 mm/min.

Monolayer 2

On a thoroughly purified surface of a buffer solution (approx. 75 ml, 10 mM NaOAc, pH 5.5; 1 mM CaCl2, 25°C; 10 mM beta-cyclodextrin (Sigma C-4767)) a monolayer of DGDG (Sigma (D4651)) is spread from a chloroform solution to a surface pressure of about 30 mN/m. After relaxation of the monolayer (evaporation of chlorofom) a solution containing approximately 30 µg (micro gram) purified enzyme is injected through the monolayer into the 75 ml subphase while surface pressure is measured continuously. Enzymatic activity is manifested through increased rate of decrease in surface pressure as DGDG is hydrolyzed into water soluble reaction products (in presence of beta cyclodextrin).

The result is considered positive for DGDGase if maximal drop in surface pressure ($d\pi/dt$) after addition of enzyme exceeds -0.5 mN/min. A number of variants of Lipolase were tested and found to have DGDGase activity, whereas the parent enzyme (Lipolase) only had very limited activity ($d\pi/dt > -0.5$ mN/min.) .

**Yeast Strain**

*Saccharomyces cerevisiae* YNG318: MATa leu2-D2 ura3-52 his4-539 pep4-D1[cir+], described in WO 97/04079 and WO 97/07205.

**Transformation of yeast strain**

The DNA fragments and the opened vectors are mixed and transformed into the yeast *Sac- charomyces cerevisiae* YNG318 by standard methods.

**Vector for yeast transformation**

pJSO026 (*S. cerevisiae* expression plasmid) is described in WO 97/07205 and in J.S.Okkels, (1996) "A URA3-promoter deletion in a pYES vector increases the expression level of a fungal lipase in Saccharomyces cerevisiae. Recombinant DNA Biotechnology III: The Integration of Biological and Engineering Sciences, vol. 782 of the Annals of the New York Academy of Sciences). It is derived from pYES 2.0 by replacing the inducible GAL1-promoter of pYES 2.0 with the constitutively expressed TPI (triose phosphate isomerase)-promoter from *Saccharomyces cerevisiae* (Albert and Karwasaki, (1982), J. Mol. Appl Genet., 1, 419-434), and deleting a part of the URA3 promoter.

**Site-directed mutagenesis**

For the construction of variants of a *H. lanuginosa* lipolytic enzyme the commercial kit, Chameleon double-stranded, site-directed mutagenesis kit can be used according to the manufacturer's instructions.

The gene encoding the lipolytic enzyme in question is inserted into the plasmid pHD414. In accord-ance with the manufacturer's instructions the ScaI site of the Ampicillin gene of pHD414 is changed to a MluI site by use of the following primer:

Primer 3: AGAAATCGGGTATCCTTTCAG.

The pHD414 vector comprising the lipolytic gene in question is then used as a template for DNA polymerase and oligos 7258 and 7770.

7258: 5'p gaa tga ctt ggt tga cgc gtc acc agt cac 3'

(Thus changing the ScaI site found in the ampicillin resistance gene and used for cutting to a MluI site).

Primer no. 7770 was used as the selection primer.

7770: 5'p tct agc cca gaa tac tgg atc aaa tc 3' (Changes the ScaI site found in the *H. lanuginosa* lipase gene without changing the amino acid sequence).

The desired mutation (e.g. in the N-terminal of the lipolytic gene or the introduction of a cystein residue) is introduced into the lipolytic gene in question by addition of an appropriate oligos comprising the desired mutation.

PCR reactions are performed according to the manufacturer's recommendations.

**Screening method**

The yeast libraries are spread on cellulose filters on SC-ura agar plates and incubated for 3-4 days at 30°C.

The filters are then transferred to the lecithin plates and incubated at 37°C for 2-6 hours. Yeast cells harboring active phospholipases will develop white clearing zones around the colonies. The positive variants can then be further purified and tested.

**Media**

SC-ura medium

| | |
|---|---|
| Yeast Nitrogen (without amino aicds) | 7.5 g |
| Succinic acid | 11.3 g |
| NaOH | 6.8 g |
| Casaminoacid (without vitamins) | 5.6 g |
| Tryptophan | 0.1 g |
| Agar, Merck | 20 g |
| Distilled water | ad 1000 ml |

Autoclaved for 20 minutes at 121°C.

From a sterile stock solution of 5% Threonine 4 ml is added to a volume of 900 ml together with 100 ml of a sterile 20% glucose.

**EXAMPLES**

**Example 1: Construction of variants with the backbone from *Humicola* lanuginosa lipase and C-terminal from *Fusarium oxysporum* phospholipase by PCR reaction**

The following variants were used as templates for the backbone from the *Humicola lanuginosa* lipase: E1A +G91A +D96W +E99K +Q249R and SPIRR +G91A +D96W +E99K +Q249R. The parent lipase was used for generating a fragment in the C-terminal without Q249R. The template for the C-terminal phospholipase was the *Fusarium oxysporum* phospholipase, cloned in the same vector as the variants of *Humicola lanuginosa* lipase.

PCR reaction 1: 4244 (SEQ ID NO: 1) as 5' primer and H7 (SEQ ID NO: 6) as 3'primer and one of the two templates mentioned above.

PCR reaction 2: FOL14 (SEQ ID NO: 3) as 5' primer and FOL15 (SEQ ID NO: 4) as 3' primer and *Humicola lanuginosa* lipase as template (no mutation in pos 249)

PCR reaction 3: FOL16 (SEQ ID NO: 5) as 5' primer and AP (SEQ ID NO: 2) as 3' primer and F.o. phospholipase as template

A PCR reaction 4 was made to create the connection between the *Humicola lanuginosa* lipase variant and the C-terminal from the phospholipase by using FOL14

(SEQ ID NO: 3) as 5' primer and AP (SEQ ID NO: 2) as 3' primer and PCR reaction 2 and 3 as template.

The final PCR was made with 4244 (SEQ ID NO: 1) as 5' primer and KBoj14 (SEQ ID NO: 7) as 3' primer and PCR re-action 1 and 4 as template (by using *Humicola lanuginosa* lipase as template in reaction 2 a possibility to omit the mutation in position 249 was created).

The final PCR fragment was used in an in vivo recombination in yeast together with pJSO026 cut with the restriction enzymes. SmaI(or BamHI) and XbaI (to remove the coding region and at the same time create an overlap of about 75 bp in each end to make a recombination event possible). This final treatment was also used in the following examples.

Primer FOL14 (SEQ ID NO: 3) and primer 15/16 are mixed oligoes to give the possibility to bind both with *Humicola lanuginosa* lipase and phospholipase templates and at the same time give possibilities for introducing the amino acids from both templates in the different positions. For some of the positions new amino acids could be introduced as well.

Primer FOL14 (SEQ ID NO: 3)

Position 205 in the *H. lanuginosa* lipase: 75% R, 25% S

Primer FOL15 (SEQ ID NO: 4) /FOL16 (SEQ ID NO: 5)

Position 256 in the *H. lanuginosa* lipase: 50% P, 50% A

Position 260 in the *H. lanuginosa* lipase: 25% R, 12.5% Q, 12.5% H, 12.5% C, 12.5% Y, 12.5% W, 12.5% stop.

The sequences of the resulting variants were determined, and were found to correspond to *Humicola lanuginosa* lipase with the following alterations. Alterations in parentheses are uncertain.

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, ( 274S)

E1A, G91A, D96W, E99K, E239C, Q249R, P256A, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G273F, (274S)

E1A, G91A, D96W, E99K, N248T, Q249R, W260Q, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, G91A, D96W, E99K, W260C, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272, G273F, (274S)

SPIRR, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

**Example 2: Production of truncated sequences**

Variants were made with stop after amino acid 269, 270, 271, 272,( 273 and 274)

The following PCR reactions were made with the following template: E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, ( 274S).

Reaction 1: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj36 (stop after 269)

Reaction 2: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj37 (stop after 270)

Reaction 3: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj38 (stop after 271)

Reaction 4: 5' primer 4244 (SEQ ID NO: 1) and 3' primer KBoj39 (stop after 272)

The sequences of the resulting variants were determined, and were found to correspond to *Humicola lanuginosa* lipase with the following alterations:

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G

E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G

**Example 3: Removal of mutations in the lid region**

G91A or E99K can be removed without loosing the phospholipase activity. The sequences of the resulting variants were determined, and were found to correspond to *Humicola lanuginosa* lipase with the following alterations:

E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

SPIRR, G91A, D96W, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S)

E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, ( 274S)

**Example 4: Doping in the C-terminal region of Humicola lanuginosa lipase to introduce phospholipase activity**

Three different libraries were constructed with possibilities for mutations in position 256 and position 263-269. At the same time possibilities for extension of the C-terminal with either 1, 2 , 3 or 4 amino acids were included.

Doping, the wt sequences are underlined:

256: P 94, A 3, T 3

263: G 87, E 4.8, A 3.8, R 3.6, Q 0.2, P 0.2

264: L 87, P 4.8, Q 3.8, V 3.6, A 0.2, E 0.2

265: I 85, T 5.6, L 2.2, S 1.6, N 1.5, F 1.4, R 0.4, K 0.4 A,P 0.1, G,D,C,H,Y 0.03, Q,E 0.01, stop 0.016

266: G 86, D 5.9, R 2, S 1.7, C 1.6, A 0.9, V 0.9, E 0.7, W 0.2, H,Y 0.1, I,L,T,F,P 0.02, Q,K 0.01, stop 0.014

267: T 86, A 6.6, S 1.9, R 0.9, N 0.9, I 0.9, K 0.9, M 0.9, P 0.9, P 0.9, G,V 0.14, D,E 0.07, L 0.03, C,Q,H,F,W,Y 0.01, stop 0.01

268: C 91, S 1.9, R 1.0, G 1.0, F 0.9, Y 0.9, L 0.04, A,N,D,H,I,P,T,V 0.01, stop 2.8

269: L 92, stop 8 (KBoj 32 (SEQ ID NO: 8) and KBoj33)/ N 86, K 2.7, D 1.8, H 1.8, I 1.8, S 1.8, T 1.9, Y 1.8, R 0.1, Q,M,E 0.06, A,C,G,L,F,P,V 0.04, stop 0.06(KBoj34)

270: stop 100 (KBoj33)/A 44, P 44, S 1.9, T 1.8, R 1.5, L 1.5, G 1.4, V 1.4, D 0.7, Q 0.7, E 0.7, H 0.7, N,C,I,K,M,F,W,Y 0.03, stop 0.03 ( KBoj 32 (SEQ ID NO: 8) and KBoj 34)

271: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

272: G 72, R 4.5, V 3.2, E 3.0, C 2.9, A 1.6, S 1.2, D 1.0, L 0.5, I,K,Y 0.15, Q,T 0.08, N,P 0.05, stop 9.2

273: F 74, L 11, S 2.8, I 2.7, V 2.7, Y 2.5, C 2.5, A,R,T 0.1, N,D,H 0.08, Q,E,K 0.01, stop 0.5

274 STOP

Library A: PCR reaction with 4244 (SEQ ID NO: 1) as 5' primer and KBoj 33 as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will be without extension.

Library B: PCR reaction with 4244 (SEQ ID NO: 1) as 5' primer and KBoj 32 (SEQ ID NO: 8) as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will most probably contain a C-terminal extension but can contain stop codons before the extension.

Library C: PCR reaction with 4244 (SEQ ID NO: 1) as 5' primer and KBoj 34 as 3' primer and E1A +G91A +D96W +E99K +Q249R or E1A +G225R as template. Variants from this library will most probably contain mutations in position 269 and a C-terminal extension but can contain stop codons before the extension.

The following variants were obtained:

Library A:

E1A +G91A +D96W +E99K +Q249R +G266D

Library B:

E1A +G91A +D96W +E99K +(R232L) +Q249R +G266S +270A
E1A +G91A +D96W +E99K +Q249R +G266S +270D +271G
E1A+ G91A+ D96W+ E99K+ Q249R+ L264G+ I265G+ G266F+ T267stop
E1A +G91A +D96W +E99K +Q249R +G266A +270P +271G
E1A +G91A +D96W +E99K +Q249R +L264P +I265F +L269stop

Library C:

E1A +G91A +D96W +E99K +Q249R +G263E +G266D +L269N +270P +271V +272G +273F
E1A +G91A +D96W +E99K +Q249R +G263A +G266S +L269N +270A +271G +272R +273F
E1A +G91A +D96W +E99K +Q249R +L264P -G266 +L269I +270P +271R +272G +273F
E1A +G91A +D96W +E99K +Q249R +G266D +L269S +270A +271G +272G +273F
E1A +D27G +G91A +D96W +E99K +Q249R +G266S +L269N +270A +271G +272G +273F
E1A +G91A +D96W +E99K +Q249R +G266D +L269N +270A
E1A +G91A +D96W +E99K +Q249R +L264P +L267Q +L269N
E1A +G91A +D96W +E99K +Q249R +G263R +I265L +L269N +270P

Example 5: For some of the above variants, the pH optimum for lipase and phospholipase was determined by using the LU and PHLU methods at various pH values. The results showed that the pH optimum phospholipase activity was in the range 4-6. The optimum for lipase activity varied from about pH 6 to about pH 10.

8 variants listed in Example 5 were analyzed for phospholipase activity by the mono layer assay described above at pH 5 and 9. The results showed that all the variants have phospholipase activity at pH 5 and 9, whereas the parent lipase

(*Humicola lanuginosa* lipase) showed no activity at pH 5 or 9. Depending on the variant, the activity at pH 5 was higher or lower than at pH 9.

A prior-art variant of *Humicola lanuginosa* lipase was found to have no phospholipase activity at pH 5: SPIRR +N94K +F95L +D96H +N101S +F181L +D234Y +I252L +P256T +G263A +L264Q.

**Example 5: Variants of *Humicola* lipase with phospholipase activity**

Variants of the parent lipase from *Humicola lanuginosa* were prepared and tested for phospholipase activity as described above. The following variants were found to have phospholipase activity, where as the parent had no phospholipase activity by the same method.

| |
|---|
| E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, ( 274S) |
| SPIRR, G91A, D96W, E99K, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S) |
| E1A, G91A, D96W, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N, 270A, 271G, 272G, 273F, (274S) |
| E1A, G91A, D96W, E99K, P256A, W260H, G263Q, L264A, I265T, G266D, T267A, L269N |
| E1A, G91A, D96W, E99K, Q249R, G266S, 270D, 271G |
| E1A, G91A, D96W, E99K, Q249R, G266D |
| E1A, G91A, D96W, E99K, Q249R, G266A, 270P, 271G |
| G266D |
| E1SPPCGRRP +E99N +E239C +Q249R +G266D |
| E1SPPCGRRP +E239C +Q249R +G266D |
| E1SPPCGRRP +L93K +E99K +E239C +Q249R +G266D |
| E1SPPCGRRP +E99K +E239C +Q249R +G266D |
| G266A |
| G266W |
| G266V |
| G263Q +L264A +I265T +G266D +T267A |
| G263F +L264A +G266S +T267E |
| E1SPPCGRRP +E239C +Q249R +G263Q +L264A +I265T +G266D +T267A |
| G266S |

| |
|---|
| G266L |
| G263A +G266A |
| G263A +G266Y |
| E1SPPCGRRP +E239C +Q249R +G266A |
| E1SPPCGRRP +E239C +Q249R +G266S |
| E1SPPCGRRP +E239C +Q249R +G263F +L264A +G266S +T267E |
| D62A + G266A |
| D62A + G266S |
| D96S + G266A |
| D96S+ G266S |
| D96S+ G266R |
| D96S+ G266W |
| D96S+ G266V |
| E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G266D |
| E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G266S |
| E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G263E+ G266S+ 270A |
| E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ L264P+ G266S |
| E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ P256T+ G266D |
| E1SPPCGRRP + G91A+ D96W+ E239C+ Q249R+ G266C+ T267P+ L269stop |
| G263D +L264I +I265N +G266E +T267GS |
| E219G +L264I +I265N +G266T +T267GL |
| E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S) |
| E1A+ G91A+ D96W+ E99K+ E239C+ Q249R+ P256A+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G +273F (+274S) |
| E1A+ G91A+ D96W+ E99K+ N248T+ Q249R+ W260Q+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S) |
| SPIRR+ G91A+ D96W+ E99K+ W260C+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272+ G273F (+274S) |
| SPIRR+ G91A+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S) |
| E1A+ G91A+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ |

| |
|---|
| 270A+ 271G+ 272G+ 273F (+274S) |
| E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G +272G +273F (+274S) |
| SPIRR+ D96W+ E99K+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S) |
| SPIRR+ G91A+ D96W+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S) |
| E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G263E+ G266D+ L269N+ 270P+ 271V+ 272G+ 273F |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G263A+ G266S+ L269N+ 270A+ 271G+ 272R+ 273F |
| E1A+ G91A+ D96W+ E99K+ Q249R+ L264P+ Δ266+ L269I+ 270P+ 271R+ 272G+ 273F |
| E1A+ G91A+ D96W+ E99K+ Q249R+ L264C+ I265N+ G266P+ T267stop |
| E1A+ G91A+ D96W+ E99K (+R232L)+ Q249R+ G266S+ 270A |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266S+ 270D+ 271G |
| E1A+ G91A+ D96W+ E99K+ Q249R+ L264F+ Δ266+ 270A+ 271G+ 272G+ 273F |
| E1A+ G91A+ D96W+ E99K+ Q249R+ L264G+ I265G+ G266F+ T267stop |
| E1A+ G91A+ D96W+ E99K+ Q249R+ L264stop |
| E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G |
| E1A+ G91A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266D |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266D |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266A+ 270P+ 271G |
| E1A+ G91A+ D96W+ E99K+ Q249R+ L264P+ I265F+ L269stop |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266D+ L269S+ 270A+ 271G+ 272G+ 273F |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266D+ L269N+ 270A |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266S+ L269N+ 270A+ 271G+ 272G+ 273F |
| E1A+ G91A+ D96W+ E99K+ Q249R+ L264P+ L267Q+ L269N |

| |
|---|
| E1A+ G91A+ D96W+ E99K+ Q249R+ G263R+ I265L+ L269N+ 270P |
| E1A+ D96W+ E99K+ P256A+ W260H+ G263Q+ L264A+ I265T+ G266D+ T267A+ L269N+ 270A+ 271G+ 272G+ 273F (+274S) |
| E1A+ G225R+ G266D |
| E1A+ G225R+ G263A+ I265V+ G266S |
| E1A+ G225R+ G263A+ T267A |
| E1SPPCGRRP+ D96S+ E239C+ Q249R+ I252M+ L264Q+ G266D |
| E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ G266D |
| E1SPPCGRRP+ D96S+ E239C+ Q249R+ G266D |
| E1SPPCGRRP+ D96S+ E239C+ Q249R+ G266C+ L267A |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266A |
| E1A+ D96M+ G106S+ G225R+ G266D |
| E1A+ D96Q+ G106S+ G225R+ G266S |
| E1A+ D96F+ G225R+ G266S |
| E1A+ D96C+ G225R+ G266T |
| E1A+ D96H+ G106S+ G225R+ G266S |
| SPIRR+ D96S+ G266D |
| SPIRR+ D96R+ G106S+ G266D |
| SPIRR+ D96I+ G106S+ G266S |
| SPIRR+ D96W+ K237R+ G266S |
| SPIRR+ G266A |
| SPIRR+ D96S+ G106S+ G225R+ G266D |
| SPIRR+ D96Q+ G106S+ G225R+ G266A |
| SPIRR+ D96Y+ G106S+ G225R+ G266N |
| SPIRR+ D96C+ G106S+ G225R+ G266T |
| SPIRR+ D96H+ T186I+ G225R+ G266S |
| E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G266D |
| E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G266S |
| E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G263E+ G266S+ 270A |
| E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ L264P+ G266S |

| |
|---|
| E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ P256T+ G266D |
| E1SPPRRP+ G91A+ D96W+ E239C+ Q249R+ G266C+ T267P+ L269stop |
| E1A+ G91A+ D96W+ E99K+ Q249R+ G266S+ T267S |
| E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ P256T+ G266S |
| E1SPPCGRRP+ E239C+ Q249R+ P256T+ G266S+ T267A |
| E1SPPCGRRP+ E239C+ Q249R+ G266D |
| E1SPPCGRRP+ G91A+ D96W+ E239C+ Q249R+ G266D |
| E1SPPRRP+ D96S+ E239C+ Q249R+ G266D |
| L259S |
| G266D |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A+ 271G+ 272G+ 273F (+274S) |
| G266E |
| G263A +G266A |
| E1SPCRPRP +E239C +Q249R +G266A |
| E1SPCRPRP +E239C +Q249R +G266S |
| D96S + G266A |
| D96S + G266S |
| D96S + G266W |
| E1SPPCGRRP +D96S +E239C +Q249R +G263D +L264I +I265N +G266E +T267GS |
| E1SPPCGRRP +D96S +E239C +Q249R +L264I +I265N +G266T +T267GL |
| D96F +G266A |
| D96F +G266S |
| E1SPPCGRRP +E99N +E239C +Q249R +G266A |
| E1SPPCGRRP + D96S +E239C +Q249R +G266A |
| E1SPPCGRRP + D96S +E239C +Q249R +G266S |
| E1SPPCGRRP + D96S +E239C +Q249R +G263F +L264A +G266S +T267E |
| V60G +D62A +S83T +R84K +D96W +G266D |
| V60G +D62A +S83T +D96W +G266D |
| V60G +D62A +S83T +D96W +G266W |

| |
|---|
| L259I |
| L259N |
| D96W +G263Q +L264A +I265T +G266D +T267A |

In the table above, (+274S) indicates that the presence of this amino acid residue at the C-terminal is uncertain. For one such variant, it was found that only a minor fraction contained this residue

Several of the above variants had a higher ratio of phospholipase (PHLU) to lipase (LU) than a prior-art enzyme from *F. oxysporum* known to have both lipase and phospholipase activity.

For some of the above variants, the pH optimum for lipase and phospholipase was determined by using the LU and PHLU methods at various pH values. The results showed that the pH optimum phospholipase activity was in the range 4-6. The optimum for lipase activity varied from about pH 6 to about pH 10.

8 variants listed in Example 5 were analyzed for phospholipase activity by the mono layer assay described above at pH 5 and 9. The results showed that all the variants have phospholipase activity at pH 5 and 9, whereas the parent lipase (*Humicola lanuginosa* lipase) showed no activity at pH 5 or 9. Depending on the variant, the activity at pH 5 was higher or lower than at pH 9.

A prior-art variant of *Humicola lanuginosa* lipase was found to have no phospholipase activity at pH 5: SPIRR +N94K +F95L +D96H +N101S +F181L +D234Y +I252L +P256T +G263A +L264Q.

The following variants of the parent lipase from *Humicola lanuginosa* may also have phospholipase activity:

| |
|---|
| D62A +S83T +D96W +G266S |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96L +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96N +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96A +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96E +E99K +G263Q +L264A +I265T +G266D +T267A +L269N |

| |
|---|
| +270A +271G +272G +273F +274S |
| G91A +D96S +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96R +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96G +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96Q +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96F +E99K +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S + |
| R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S |
| R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S + |
| R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96F +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96W +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96F +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96W +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |

**Example 6: Variants of *Rhizomucor* lipase with phospholipase activity**

The following two variants of the parent lipase from *Rhizomucor miehei* were prepared and tested for phospholipase activity as described above. The variants were found to have phospholipase activity, where as the parent had no phospholipase activity by the same method.

G266N

G266V

**Example 7: Variants of *Humicola* lipase with increased specificity for long-chain fatty acids**

Variants of the parent lipase from *Humicola lanuginosa* were prepared and tested for their hydrolytic activity on two triglyceride substrates with different chain length: tributyrin ($C_{4:0}$) and triolein ($C_{18:1}$). The tests were done at pH 9 by the LU and SLU methods described above. The following variants were found to have a higher ratio of triolein activity to tributyrin activity than the parent enzyme (*Humicola lanuginosa* lipase):

| |
|---|
| E1SPIRPRP +G91A +D96N +E99K +Q249R |
| E1SPCRPRP+ S83T+ N94K+ D96L+ E239C+ Q249R |
| G266D |
| E1SPIRPRP +D62A +E99K +Q249R |
| E1SPIRPRP +D62G +E99K +Q249R |
| E1SPIRPRP +D62V +E99K +Q249R |
| E1SPIRPRP +R84W +E99K +Q249R |
| E1SPIRPRP +R84K +E99K +Q249R |
| E1SPIRPRP + K98D +E99K +Q249R |
| E1SPIRPRP + E99K +Q249R + 270PGLPFKRV |
| E1SPPCGRRP + E99N +N101S +T231K +R232G +D234G +E239C +Q249R |
| E1SPIRPRP +E99K +Q249R + 270PWPARLGRL |
| L93K +D96G |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A+ 271G+ 272G+ 273F (+274S) |
| E1SPCRPRP +V60G +E99N +S119G +R209P +E239C +Q249R |
| G266A |
| G266E |
| G266V |
| G263Q +L264A +I265T +G266D +T267A |
| G266L |
| G263A +G266A |
| E1SPCRPRP +E239C +Q249R +G266A |

| |
|---|
| E1SPCRPRP +E239C +Q249R +G266S |
| D96S + G266A |
| D96S + G266S |
| D96S + G266W |
| L264I +I265N +G266T +T267GL |
| E1SPPCGRRP +D96S +E239C +Q249R +L264I +I265N +G266T +T267GL |
| D96F +G266A |
| D96F +G266S |
| E1SPPCGRRP +E99N +E239C +Q249R +G266A |
| E1SPPCGRRP + D96S +E239C +Q249R +G266A |
| E1SPPCGRRP + D96S +E239C +Q249R +G266S |
| D62A + S83T |
| E1SPPCGRRP +K98D +E99N +E239C +Q249R |
| T231R +N233R +270CP |
| E1SPPCGRRP +E99N +E239C +Q249R +270MD |
| E1SPPCGRRP + D62A +S83T +E99N +E239C +Q249R |
| D62A +S83T + G91A +E99K +T231R +N233R +Q249R |
| V60G +D62A +S83T +R84K +D96W +G266D |
| L259N |
| L259R |
| L259M |
| L259Q |
| SPPCGRRP(-E) +R84W +E99N +N101S +E239C +Q249R |
| R84W +G91A +E99K +T231R +N233R +Q249R |
| Y21I |
| Y21V |
| SPIRPRP(-E) +R84L +E99K +Q249R |
| Y21C |
| SPIRPRP(-E) +D62 +E99K +Q249R |
| D96W +G263Q +L264A +I265T +G266D +T267A +L269N +A270 +G271 +G272 +F273 |

| |
|---|
| +S274. |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |

The following variants of the parent lipase from *Humicola lanuginosa* may also have an increased specificity for long-chain fatty acids:

| |
|---|
| SPIRPRP(-E) +V60R +D62V +L93K +E99K +Q249R |
| SPIRPRP(-E) +D62V +E99K +Q249R |
| SPIRPRP(-E) +E99K +Q249R +P256D |
| SPIRPRP(-E) +D62V +E99K +Q249R +P256D |
| SPIRPRP(-E) +D62V +E99K +Q249R +P256S |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96L +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96N +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96A +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96E +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96S +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96R +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96G +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96Q +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S |
| G91A +D96F +E99K +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S |
| R84W +G91A +D96W +E99K +G263Q +L264A +I265T +G266S +T267A +L269N |

| |
|---|
| +270A +271G +272G +273F +274S |
| R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266S +T267A +L269N +270A +271G +272G +273F +274S |
| R84W +G91A +D96F +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270A +271G +272G +273F +274S |
| SPPCGRRP(-E) +V60G +D62E +S83T +R84K +E99N +N101S +E239C +Q249R |
| V60G +D62E +S83T +R84K +G91A +E99K +T231R +N233R +Q249R |

**Example 8: Variants of *Fusarium* lipase with increased specificity for long-chain fatty acids**

Variants of the parent lipase from *Fusarium oxysporum* were prepared and tested as in the previous example. The following variants were found to have a higher ratio of triolein activity to tributyrin activity than the parent enzyme:

Y23S

Y260L

The following variants of the parent lipase from *Fusarium oxysporum* may also have an increased specificity for long-chain fatty acids:

R80H +S82T

S82T +A129T

**Example 9: Variants of *Rhizomucor* lipase with increased specificity for long-chain fatty acids**

The following variants of the parent lipase from *Rhizomucor miehei* may have an increased specificity for long-chain fatty acids:

Y260W

Y28L

Y28C +H217N

**Example 10: Variants of *Humicola* lipase with increased specificity for short-chain fatty acids**

Variants of the parent lipase from *Humicola lanuginosa* were prepared and tested as in the previous example. The following variants were found to have a higher ratio of tributyrin activity to triolein activity (a lower SLU/LU ratio) than the parent enzyme:

| |
|---|
| SPIRPRP(-E) +E99K +R195Q +R209E +Q249R |
| N101R +R195Q +R209E +L259S +Y261D |
| N101R +R195Q +R209E +L259S |
| N101R +L259S +Y261D |
| N101R +L259S |
| Y261D |
| L259S |
| SPIRPRP(-E) +E99K +N101R +Q249R |
| G263D +L264I +I265N +G266E +T267GS |
| Y261I |
| D234R |
| Y261K |

The following variants of the parent lipase from *Humicola lanuginosa* may also have a higher ratio of tributyrin activity to triolein activity:

| |
|---|
| N101R,R195Q,R209E,L259S,Y261D |
| N101R,R195Q,R209E,L259S |
| N101R,L259S,Y261D |
| N101R,L259S |

**Example 11: Variants of *Fusarium* lipase with increased specificity for short-chain fatty acids**

Variants of the parent lipase from *Fusarium oxysporum* were prepared and tested as in the previous example. The following variants were found to have a higher ratio of tributyrin activity to triolein activity than the parent enzyme:

Y23W
Y260D
Y260R
Y260C
Y260N

**Example 12: Variants of *Rhizomucor* lipase with increased specificity for short-chain fatty acids**

The following variants of the parent lipase from *Rhizomucor miehei* may have an increased specificity for short-chain fatty acids:

Y260C
Y260G
Y260V

**Example 13: Variants of *Humicola* lipase with DGDGase activity**

Variants of the parent lipase from *Humicola lanuginosa* were prepared, and the hydrolytic activity towards DGDG (di-galactosyl-di-glyceride) was determined as described above. The following variants were found to have DGDGase activity, whereas the parent lipase gave a negative result.

| |
|---|
| D96W +G263Q +L264A +I265T +G266D +T267A |
| G263Q +L264A +I265T +G266D +T267A |
| D96W +G263Q +L264A +I265T +G266D +T267A +L269N +270AGGFS |
| G91A +D96W +E99K +G263Q +L264A +I265T +G266D +T267A +L269N +270AGGFS |
| D96F +G266S |

**Example 14: Variants of *Humicola* lipase with increased pH optimum**

Variants of the parent lipase from *Humicola lanuginosa* were prepared, and the lipase activity was measured by the LU method at pH 7 and 9. The following variants were found to have a higher ratio of activity at pH 9 to activity at pH 7 than the parent lipase:

R84L
R84W
Y21I
Y21V
Y261I

**Example 15: Variants of *Humicola* lipase with decreased pH optimum**

Variants of the parent lipase from *Humicola lanuginosa* were prepared, and the lipase activity was measured by the LU method at pH 7 and 9. The following variants were found to have a lower ratio of activity at pH 9 to activity at pH 7 than the parent lipase:

Y261D
G266D/E
Y261W

**Example 16: Use of *Humicola* lipase variants in degumming of vegetable oil**

Rapeseed oil was treated with two variants of the lipase from *Humicola lanuginosa*, essentially as described in Example 6 of WO 98/18912 (Novo Nordisk).

One variant was tested at an enzyme dosage of 0.6 mg of enzyme protein per kg of oil. Results of tests at various pH and temperatures showed optimum performance at pH 5.7, 35-45°C, where a final P content of 4 ppm was reached. A separate experiment at 45°C, pH 6 showed that a final P content of 4 ppm could be reached at an enzyme dosage as low as 0.15 mg/kg.

A similar experiment with another *Humicola lanuginosa* lipase variant showed optimum performance at 40°C, pH 5.0-5.5. The enzyme dosage was 0.3 mg/kg.

A degumming experiment was made with a third *Humicola lanuginosa* lipase variant, using rape seed oil at 45°C, pH 5, 1.8 mg enzyme/kg oil. For comparison, a similar experiment was made with the parent lipase (*Humicola lanuginosa* lipase) at 18 mg/kg. The results showed that good degumming (<10 ppm residual P content) was obtained in 3.4 hours with the variant.

The parent lipase (*Humicola lanuginosa* lipase) was found to have very little degumming effect, even at 10 times higher enzyme dosage.

**Example 17: Use of lipase variants in baking**

A variant of the lipase from *Humicola lanuginosa* was evaluated in baking tests as follows.

Doughs were prepared from Meneba flour according to the European straight dough method (ABF-SP-1201.01) with 40 ppm of ascorbic acid. Various combinations of additives at the following dosages were used: the lipase variant at 0, 0.25, 0.5 or 1.5 mg/kg; phospholipid (lecithin) at 0 or 10 g/kg; and endo-amylase at 0 or 750 MANU/kg.

The endo-amylase was maltogenic amylase from *B. stearothermophilus* (tradename Novamyl®). One MANU (Maltogenic Amylase Novo Unit) is defined as the amount of enzyme required to release one μmol of maltose per minute at a concentration of 10 mg of maltotriose substrate per ml of 0.1 M citrate buffer, pH 5.0 at 37°C for 30 minutes.

After baking, the loaves were cooled, and the loaf volume, crumb firmness and softness were evaluated after approximately 2 hours. The evaluation was repeated after 1, 3 and 7 days storage at 22°C wrapped in double plastic bags.

Firmness of crumb was measured using a texture analyzer TA-XT2 from Stable Micro Systems (probe diameter 40 mm).

Softness in gram was measured as the force needed to press a probe 6.25 mm into a crumb of a 25 mm thick slice of bread (25 % penetration).

The results showed that the addition of 1.5 mg of the variant increased the loaf volume. The results for firmness and elasticity show that the variant gives significantly softer crumb and significantly better elasticity from day 0 to day 7.

**Example 18: Use of lipase variants for dough stability in baking**

A variant of the *Humicola lanuginosa* lipase was evaluated in a baking trial to evaluate its tolerance towards extended proofing of the dough.

Doughs were prepared from Pelikan flour according to the European straight dough method (347-SP-1217) with 30 ppm ascorbic acid, fungal α-amylase (10 FAU of Fungamyl), and pentosanase (100 FXU of Pentopan Mono). Dosages of 0.2, 0.4, and 0.6 mg enzyme protein/kg flour of the variant were compared with 1000 LU of the parent lipase.

The doughs were made into rolls. Half of the rolls were proofed for 45 minutes (normal proofing) and the other half for 70 minutes (over proofing).

After baking the bread was cooled, and the volume and the standing of the rolls were evaluated after approximately 2 hours. The standing is a measure of the shape of the rolls and is defined as the height of 10 rolls divided by the width of 10 rolls, which means that nice round loaves have a high standing value, whereas flat rolls have a low standing value.

The results showed that at normal proofing time the volume of 0.4 and 0.6 mg of the variant were better than that of the parent lipase, and the standing of the rolls were better for the variant at all dosages than for the parent lipase. When the rolls were over proofed, both volume and standing was better for the variant at all dosages than for the parent lipase.

**Example 19: Effect of lipase variants on off-odor development**

The development of off-odor from lipases with different chain-length specificity was evaluated in whole milk. The developed butyric acid/sour odor was evaluated by sniffing the samples after heating.

25 ml whole milk was placed in 100 ml blue cap flasks (with caps) in a 32°C water bath. Of each of the lipases listed below, 0.2 mg enzyme protein per litre milk was added to the flasks. The temperature was raised to 45°C, and evaluation took place after 15 and 105 minutes.

The lipases tested were *Humicola lanuginosa* lipase and variants thereof. For each lipase, the chain-length specificity is expressed as the ratio of activities on triolein (SLU) and tributyrin (LU).

Three persons evaluated the samples and agreed on the ranking shown below

+ Detectable smell

++ Clear and characteristic butyric acid and/or sour odor

+++ Strong butyric acid and/or sour odor

Three variants of *Humicola lanuginosa* lipase having a higher SLU/LU ratio than *Humicola lanuginosa* lipase were found to have less malodor than the parent lipase.

**Example 20: Effect of lipase variants on malodour on textile after washing**

Soiling:

Cotton textile was soiled with a dairy product as described here. 50 mg of butter was applied over an area of approximately 30 $cm^2$ in an even spot. The soiled textile was aged for 24 hours at ambient conditions.

Washing procedure:

Washing of the soiled textile was done in a Terg-O-tometer using a commercial detergent(5 g/l) with and without lipase(1250 and 5000 LU/l). The washing was done at 30°C for 20 min at 100 rpm. After washing the swatches were left overnight to dry at ambient conditions.

Sensory analysis:

Next day, the malodour was assessed by a sensory panel consisting of at least 10 trained assessors. Samples were kept in tight glass jars and left at least 30 minutes between every evaluation for accumulation of malodour. Swatches were taken out and the malodour assessed on the textile. The butyric acid malodour was scored according to the scale below. As a reference the sample washed without lipase was used.

0. Fainter smell than reference
1. Same as reference
2. Slightly stronger than reference
3. Definitely stronger than reference
4. Stronger than 3.

Variants of *Humicola lanuginosa* lipase with an increased ratio of triolein/tributyrin activities (increased SLU/LU ratio) were found to give a fainter smell from butter stains than the parent enzyme (*Humicola lanuginosa* lipase). A separate washing experiment showed that the variants, like the parent enzyme, were effective in the removal of lard stains.

Alternative methods

The intensity of butyric acid from dairy stains on fabric can also be evaluated by instrumental analysis:

1. By Head Space Gas Chromatography, or
2. By extraction of the odours from fabric followed by Gas Chromatography

**Example 21: Effect of lipase variants on odour of bread baked with butter**

Six variants of the lipase from *Humicola lanuginosa* were prepared and were evaluated in bread baked by the European straight dough procedure (347-SP-1217) with addition of 3% butter. 0.2 mg enzyme protein/kg flour was used for each of the variants.

The chain-length specificity of the variants was also determined by measuring the triolein/tributyrin activity ratio (SLU/LU described above). The parent lipase from *Humicola lanuginosa* and a prior-art lipase with phospholipase acivity from *Fusarium oxysporum* were also tested for comparison.

The results are summarized below:

+ detectable smell
++ clear and characteristic butyric acid and/or sour odour
+++ strong butyric acid and/or sour odour

| | SLU/LU | Rating |
|---|---|---|
| Variants of the | 2.7 | (+) |
| | 3 | no effect |
| | 7 | no effect |

| | | |
|---|---|---|
| invention | 28 | no effect |
| | 70 | no effect |
| Parent lipase | 1.2 | ++ |
| Prior-art lipase | 1.1 | +++ |
| Control (no lipase) | - | no effect |

The results indicate that that lipase variants with a SLU/LU ratio at 3 or above (i.e. a high specificity for long-chain fatty acids) give no unpleasant odour in bread baking even with butter in the recipe.

## CLAIMS

1. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having an alcohol binding site having a glycerol part with an sn2 position,

c) in the parent lipolytic enzyme selecting at least one amino acid residue which comprises at least one atom within 10 Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride in a three-dimensional structure of the parent lipolytic enzyme and the substrate,

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

f) preparing the variant resulting from steps b-d,

g) testing the activity of the variant on the selected ester bond,

h) selecting a variant having an altered activity on the selected ester bond, and

i) producing the selected variant.

2. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having a structure comprising a catalytic triad consisting of an active Ser, an active Asp and an active His residue,

c) in the parent lipolytic enzyme selecting at least one amino acid residue comprising at least one atom belonging to a set E defined by the following steps:

i) aligning the structure of the lipolytic enzyme with *Rhizomucor miehei* lipase structure 4TGL comprising a catalytic triad and an inhibitor phosphorus atom (4TGL-inhP), so as to minimize the sum of squares

of deviation between atoms of the catalytic triads of the two structures,

ii) defining a set A consisting of atoms of the lipolytic enzyme inside a sphere of radius 18 Å with center at 4TGL-inhP,

iii) forming a first plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of the active Asp residue of the parent lipolytic enzyme and defining a set B as a subset of set A consisting of atoms on the same side of the first plane as the Cα atom of the active His residue of the parent lipolytic enzyme,

iv) forming a second plane defined by 4TGL-inhP, the Cα atom of the active Ser residue of the parent lipolytic enzyme, and the Cα atom of the active His residue of the parent lipolytic enzyme and defining a set C as a subset of set A consisting of atoms on the opposite side of the second plane from the Cα atom of the active Asp residue of the parent lipolytic enzyme,

v) forming a set D consisting of atoms belonging to the union of sets B and C, and having a solvent accessibility of 15 or higher, and

vi) forming set E consisting of amino acid residues in the structure which comprise an atom belonging to set D or an atom belonging to the union of sets B and C and located less than 3.5 Å from an atom belonging to set D,

d) making alterations each of which is an insertion, a deletion or a substitution of the selected amino acid residues,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than d),

f) preparing the variant resulting from steps d) - f), and

g) testing the activity of the variant on the selected ester bond,

h) selecting a variant having an altered activity on the selected ester bond, and

i) producing the selected variant.

3. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having an active site comprising an active His residue,

c) in the amino acid sequence of the parent lipolytic enzyme selecting at least one amino acid residue at the C-terminal side of the active His residue,

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than b),

f) preparing the variant resulting from steps b-d,

g) testing the activity of the variant on the selected ester bond,

h) selecting a variant having an altered activity on the selected ester bond, and

i) producing the selected variant.

4. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme,

c) selecting at least one amino acid residue among 10 amino acid residues at the C-terminal,

d) making alterations each of which is an insertion, a deletion or a substitution of the selected amino acid residues,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the variant resulting from steps c) - e),

g) testing the activity of the variant on the selected ester bond,

h) selecting a variant having an altered activity on the selected ester bond, and

i) producing the selected variant.

5. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme having a lid,

c) selecting at least one amino acid residue in the lid,

d) making alterations each of which is an insertion, a deletion or a substitution of the selected amino acid residues,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the variant resulting from steps c) - e),

g) testing the activity of the variant on the selected ester bond,

h) selecting a variant having an altered activity on the selected ester bond, and

i) producing the selected variant.

6. The method of any of claims 1-5 wherein the lipolytic enzyme is native to an eukaryote, preferably to a fungus, and most preferably belongs to the *Humicola* family or the *Zygomycetes* family.

7. A method of producing a lipolytic enzyme variant comprising:

a) selecting a substrate and an ester bond of interest,

b) selecting a parent lipolytic enzyme from the *Humicola* family or the *Zygomycetes* family,

c) selecting at least one amino acid residue corresponding to any of amino acids 20-25, 56-64, 81-85 and 255-269 in the *Humicola lanuginosa* lipase

d) making alterations each of which is an insertion, a deletion or a substitution of the amino acid residue,

e) optionally, making alterations each of which is an insertion, a deletion or a substitution of an amino acid residue at one or more positions other than c),

f) preparing the variant resulting from steps b-e,

g) testing the activity of the variant on the ester bond in the substrate, and

h) selecting a variant having an altered activity on the ester bond.

8. The method of claim 7 wherein the parent lipolytic enzyme is the lipase of *Humicola lanuginosa* strain DSM 4109.

9. The method of any of claims 1-8 wherein the altered activity is a lower activity towards a $C_4$-$C_8$ acyl bond in a triglyceride, or a lower ratio of activity towards a $C_4$-$C_8$ acyl bond in a triglyceride and a $C_{16}$-$C_{20}$ acyl bond in a triglyceride.

10. The method of claim 9 wherein the parent lipolytic enzyme belongs to the *Humicola* family or the *Zygomycetes* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109, and the selected amino acid residues comprise an amino acid corresponding to Y21, E56, D57, V60, G61, D62, R81, S83, R84, L259, Y261 or G266 in the *Humicola lanuginosa* lipase.

11. The method of any of claims 1-8 wherein the altered activity is a lower activity towards a $C_{16}$-$C_{20}$ acyl bond in a triglyceride, or a lower ratio of activity towards a $C_{16}$-$C_{20}$ acyl bond in a triglyceride and a $C_4$-$C_8$ acyl bond in a triglyceride.

12. The method of any of claims 1-8 wherein the altered activity is a higher phospholipase activity.

13. The method of claim 12 wherein the parent lipolytic enzyme has a phospholipase activity below 50 PHLU/mg and/or a ratio of phospholipase activity to lipase activity below 0,1 PHLU/LU.

14. The method of claim 12 or 13 wherein the parent lipolytic enzyme belongs to the *Humicola* family or the *Zygomycetes* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109, and the selected amino acid residues comprise an amino acid corresponding to R81, R84, S85, or 263-267 (e.g. G266 or T267) in the *Humicola lanuginosa* lipase.

15. The method of any of claims 12-14 wherein the alterations comprise insertion of a peptide extension at the C-terminal, preferably comprising 1-5 amino acid residues, the first preferably being A, P or D, the second (if present) preferably being V, G or

R, the third (if present) preferably being V, G or R, the fourth (if present) preferably being F, and the fifth (if present) preferably being S.

16. The method of any of claims 1-8 wherein the altered activity is a higher hydrolytic activity on a digalactosyl-diglyceride.

17. The method of claim 16 wherein the parent lipolytic enzyme belongs to the *Humicola* family or the *Zygomycetes* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109, and the selected amino acid residues comprise an amino acid corresponding to 21, 23, 26, 57, 62, 81, 83, 84, 85, 266, 267 or 269 in the *Humicola lanuginosa* lipase.

18. A method of preparing a lipolytic enzyme variant for use in baking, which method comprises:

a) selecting a parent lipolytic enzyme,

b) making at least one alteration which is an insertion, a deletion or a substitution of an amino acid residue in the lipolytic enzyme to obtain a lipolytic enzyme variant,

c) screening for a lipolytic enzyme variant which compared to the parent lipolytic enzyme has:

i) a higher ratio selectivity for long-chain fatty acyl groups,

ii) a higher activity on digalactosyl diglyceride, and

iii) a higher phospholipase activity, and

d) preparing the lipolytic enzyme variant.

19. The method of claim 18 wherein the parent lipolytic enzyme and the amino acid to be altered are selected as defined in any of claims 1-8.

20. A method of preparing a lipolytic enzyme variant for use in baking, which method comprises

a) subjecting a DNA sequence encoding a lipolytic enzyme to random mutagenesis,

b) expressing the mutated DNA sequence obtained in step (a) in a host cell, and

c) screening for host cells expressing a lipolytic enzyme variant which compared to the parent lipolytic enzyme has:

i) a higher ratio selectivity for long-chain fatty acyl groups,

ii) a higher activity on digalactosyl diglyceride, and

iii) a higher phospholipase activity, and

d) preparing the lipolytic enzyme expressed by the host cells.

21. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having an alcohol binding site having a glycerol part with an sn2 position, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue, at a position which in a three-dimensional structure of the parent lipolytic enzyme and a substrate is within 10 Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride, and

b) has an altered activity on an ester bond in the substrate.

22. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having a lid, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of an amino acid residue in the lid,

b) has an altered activity on an ester bond in the substrate.

23. A lipolytic enzyme which is a variant of a parent lipolytic enzyme having an active site comprising an active His residue, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of at least one amino acid residue at the C-terminal side of the active His residue,

b) has an altered activity on an ester bond in a substrate.

24. A lipolytic enzyme which is a variant of a parent lipolytic enzyme, which variant:

a) comprises an alteration which is an insertion, a deletion or a substitution of at least one amino acid within 10 amino acid residues of the C-terminal,

b) has an altered activity on an ester bond in a substrate.

25. The lipolytic enzyme of any of claims 21-24 wherein the parent lipolytic enzyme is native to an eukaryote, preferably to a fungus, and most preferably belongs to the *Zygomycetes* family.

26. The lipolytic enzyme of claim 25 wherein the parent lipolytic enzyme belongs to the *Humicola* family or the *Zygomycetes* family, preferably the lipase of *Humicola lanuginosa* strain DSM 4109.

27. A lipolytic enzyme which:

a) is a polypeptide having an amino acid sequence which has at least 80 % homology with a reference lipolytic enzyme of the *Humicola* family or the *Zygomycetes* family;

b) compared to said reference lipolytic enzyme comprises an amino acid alteration which is:

i) a substitution, deletion or insertion at a position corresponding to A20, Y21, G23, K24, N25, V63, R81, G82, R84, A257, W260, Y261, F262 or G266 in the *Humicola lanuginosa* DSM 4109 lipase;

ii) a substitution of an amino acid corresponding to C268 or L269 in said lipase;

iii) a substitution corresponding to V60G, D62E, L93K, L97Q, K98E,F, E99D, P256A, G263E,Q,R,F,N, L264A,C,P,F,G,V,I, I265L,N,F or T267A,Q,P,S,V,E in said lipase;

iv) an insertion corresponding to T267GS or T267GL in said lipase;

v) a peptide extension at the C-terminal which is A, P, MD, CP, AG, DG, AGG, PVGF, AGRF, PRGF, AGGF or AGGFS;

vi) a peptide extension at the C-terminal of 40-50 amino acids; or

vii) a truncation of 1, 2, 3, 4, 5 or 6 amino acids at the C-terminal; and

c) has an altered activity on an ester bond in a substrate compared with the reference lipolytic enzyme.

28. The lipolytic enzyme of claim 27 wherein the amino acid alteration corresponds to R84K,L,W, W260H,Q,C, G266A,C,D,N,L,I,S,T,P,V,F,W,E,K,R,Y or L269N,I,S.

29. The lipolytic enzyme of claim 27 or 28 which further comprises at least one further amino acid alteration which is a substitution, a deletion or an insertion corresponding to any of positions 22, 56-59, 61, 64, 83, 85, 91, 94, 249, 255 or 259 preferably S83T, G91A, N94D, D96S,W,F,G, Q249R or L259N,R,S,M,Q.

30. The lipolytic enzyme of any of claims 27-29 which further comprises a substitution corresponding to D62A,G,V, K98D, E99K, P256T, G263A and/or I265T,G,V.

31. The lipolytic enzyme of any of claims 27-30 which comprises a peptide extension at the N-terminal.

32. The lipolytic enzyme of any of claims 27-31 wherein the reference lipolytic enzyme is the lipase from *Humicola lanuginosa*.

33. The lipolytic enzyme of any of claims 27-31 wherein the reference lipolytic enzyme is the lipase from *Rhizomucor miehei*.

34. The lipolytic enzyme of any of claims 27-31 wherein the reference lipolytic enzyme is the lipase from *Fusarium oxysporum*.

35. A lipolytic enzyme which:

a) is a polypeptide having an amino acid sequence which has at least 80 % homology with a reference enzyme which is the lysophospholipase from *Aspergillus foetidus,* the ferulic acid esterase from *Aspergillus niger,* the ferulic

acid esterase from *Aspergillus tubigensis* or phospholipase A1 from *Aspergillus oryzae*,

b) compared to said reference enzyme comprises an amino acid alteration which is a substitution, deletion or insertion at a position corresponding to 20-25, 56-64, 81-85, 91-98, 255-257 or 259-269 in the *Humicola lanuginosa* lipase, and

c) has an altered activity on an ester bond in a substrate compared with the reference enzyme.

36. The lipolytic enzyme of claim 35 wherein the alteration is at a position corresponding to Y21, E56, D57, V60, G61, D62, S83, R84, G91, L93, N94, D96, L97, K98, E99, P256, W260, Y261, G263, L264, I265, G266, T267 or L269 in the *Humicola lanuginosa* lipase

37. The lipolytic enzyme of claim 35 or 36 wherein the alteration is an extension or a truncation at the C-terminal, preferably by 1-5 amino acids.

38. The lipolytic enzyme of any of claims 27-37 wherein the altered activity is a lower activity towards a $C_4$-$C_8$ acyl bond in a triglyceride, or a lower ratio of activity towards a $C_4$-$C_8$ acyl bond in a triglyceride and a $C_{16}$-$C_{20}$ acyl bond in a triglyceride.

39. The lipolytic enzyme of claim 38 which comprises an amino acid alteration at a position corresponding to Y21, E56, D57, V60, G61, D62, R81, S83, R84, L259, Y261 or G266 in the *Humicola lanuginosa* lipase.

40. The lipolytic enzyme of any of claims 27-37 wherein the altered activity is a lower activity towards a $C_{16}$-$C_{20}$ acyl bond in a triglyceride, or a lower ratio of activity towards a $C_{16}$-$C_{20}$ acyl bond in a triglyceride and a $C_4$-$C_8$ acyl bond in a triglyceride.

41. The lipolytic enzyme of any of claims 27-37 wherein the altered activity is a higher phospholipase activity.

42. The lipolytic enzyme of claim 41 which comprises an amino acid alteration at a position corresponding to R81, R84, S85, G263, L264, I265, G266, T267 or L269 in the *Humicola lanuginosa* lipase, preferably a substitution corresponding to G263A,E,Q,R; L264A,C,P,Q; I265L,N,T; G266A, C, D, N, L, I, S, T, P, V or T267A,Q or L269N.

43. The lipolytic enzyme of claims 41 or 42 which has a phospholipase activity greater than 0.1 nmol/min in a monolayer assay at pH 5 as described herein and/or a phospholipase activity greater than 100 PHLU/mg (preferably greater than 500 PHLU/mg) and/or a ratio of phospholipase activity to lipase activity greater than 0.1 PHLU/LU (preferably greater than 0.5 PHLU/LU).

44. The lipolytic enzyme of any of claims 41-43 wherein the parent lipolytic enzyme has a phospholipase activity below 50 PHLU/mg and/or a ratio of phospholipase activity to lipase activity below 0,1 PHLU/LU.

45. The lipolytic enzyme of any of claims 41-44 which comprises a peptide extension at the C-terminal, preferably comprising 1-5 amino acid residues, the first preferably being A, P or D, the second (if present) preferably being V, G or R, the third (if present) preferably being V, G or R, the fourth (if present) preferably being F, and the fifth (if present) preferably being S.

46. The lipolytic enzyme of any of claims 41-45 which comprises a deletion of amino acid residues at positions corresponding to positions C268 and L269 in the lipase of *Humicola lanuginosa* strain DSM 4109.

47. The lipolytic enzyme of any of claims 27-37 wherein the altered activity is a higher hydrolytic activity on a digalactosyl-diglyceride.

48. The lipolytic enzyme of claim 47 which comprises an amino acid alteration at a position corresponding to Y21, G23, N26, D57, D62, R81, S83, R84, S85, G266, T267 or L269 in the *Humicola lanuginosa* lipase, preferably comprising two or more

such alterations, most preferably further comprising at least one alteration in the lid region

49. The lipolytic enzyme of any of claims 27-48 which comprises an alteration in the lid which is a substitution of a negatively charged amino acid residue with a neutral or positively charged amino acid residue, or a substitution of a neutral amino acid residue with a positively charged amino residue.

50. The lipolytic enzyme of claim 49 which comprises an alteration in the lid at a position corresponding to position G91, D96 and/or E99 in the *Humicola lanuginosa* lipase, preferably a substitution which is G91A, D96S,W,F or E99K.

51. A lipolytic enzyme which is a variant of a parent lipase derived from *Humicola lanuginosa* strain DSM 4109 comprising the alterations E1E,D,A+ G91G,A,S,T+ N94N,D+ D96D,G,F,W+ E99E,K+ G225G,R,K+ G263Q,N+ L264L,A,V+ I265I,T,S+ G266G,A,V,S,D,E+ T267T,A,V+ L269L,I,N,Q.

52. The lipolytic enzyme of claim 51 which further comprises SPIRR as a peptide extension at the N-terminal and/or AGGF or AGGFS as a peptide extension at the C-terminal.

53. A DNA sequence encoding the lipolytic enzyme of any of claims 27-52.

54. A vector comprising the DNA sequence of claim 53.

55. A transformed host cell harboring the DNA sequence of claim 53 or the vector of claim 54.

56. A method of producing the lipolytic enzyme of any of claims 27-52 comprising

a) cultivating the cell of claim 55 so as to express and preferably secrete the lipolytic enzyme, and

b) recovering the lipolytic enzyme.

57. A process for preparing a dough or a baked product prepared from the dough which comprises adding the lipolytic enzyme of any of claims 27-52 to the dough, wherein the lipolytic enzyme preferably has phospholipase and/or digalactosyl diglyceride activity.

58. The process of claim 57 which further comprises adding to the dough an endo-amylase and/or a phospholipid.

59. The process of claim 57 or 58 wherein the endo-amylase is from *Bacillus*, and is preferably a maltogenic amylase from *B. stearothermophilus*,

60. A process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the lipolytic enzyme of any of claims 41-46, 51 or 52 so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

61. A process for improving the filterability of an aqueous solution or slurry of carbohydrate origin which contains phospholipid, which process comprises treating the solution or slurry with the lipolytic enzyme of any of claims 41-46, 51 or 52, wherein the solution or slurry preferably contains a starch hydrolysate, particularly a wheat starch hydrolysate.

62. A detergent composition comprising a surfactant and the lipolytic enzyme of any of claims 27-52, wherein the lipolytic enzyme preferably has a specificity for long-chain fatty acids corresponding to a ratio of SLU to LU above 3.

63. A method of enhancing the flavor of a food product containing milk fat, comprising treating the food product with the lipolytic enzyme of any of claims 27-52 so as to release free fatty acids, wherein the lipolytic enzyme preferably has a specificity for short-chain fatty acids corresponding to a ratio of SLU to LU below 0.5, more preferably below 0.2, e.g. below 0.1.

64. A method of preparing a dough or a baked product prepared from the dough, comprising:

a) testing at least one lipolytic enzyme for its hydrolytic activity towards a $C_4$-$C_8$ acyl bond in a triglyceride, a $C_{16}$-$C_{20}$ acyl bond in a triglyceride, digalactosyl diglyceride and a phospholipid,

b) selecting a lipolytic enzyme having hydrolytic activity towards digalactosyl diglyceride and the phospholipid, and having a ratio of activity towards the $C_{16}$-$C_{20}$ acyl bond and the $C_4$-$C_8$ acyl bond which corresponds to a SLU/LU ratio of at least 3, and

c) adding the selected lipolytic enzyme to the dough.

```
          1                                                  50
  rhimi   SIDGGIRAAT SQEINELTYY TTLSANSYCR TVIPGA...T WDC..IHCDA
  rhidl   SDGGKVVAAT TAQIQEFTKY AGIAATAYCR SVVPGN...K WDC..VQCQK
  SP400   -------EVS QDLFNQFNLF AQYSAAAYCG KNNDAPAGTN ITCTGNACPE
    Pcl   -------DVS TSELDQFEFW VQYAAASYYE ADYTAQVGDK LSCSKGNCPE
FoLnp11   -----AVGVT TTDFSNFKFY IQHGAAAYC. .NSEAAAGSK ITCSNNGCPT

          51                                                 100
  rhimi   TE..DLKIIK TWS.TLIYDT NAMVARGDSE KTIYIVFRGS SSIRNWIADL
  rhidl   WV.PDGKIIT TFT.SLLSDT NGYVLRSDKQ KTIYLVFRGT NSFRSAITDI
  SP400   VEKADATFLY SFEDSGVGDV TGFLALDNTN KLIVLSFRGS RSIENWIGNL
    Pcl   VEATGATVSY DFSDSTITDT AGYIAVDHTN SAVVLAFRGS YSVRNWVADA
FoLnp11   VQGNGATIVT SFVG.SKTGI GGYVATDSAR KEIVVSFRGS INIRNWLTNL

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  rhidl   VFNFSDY.KP VKGAKVHAGF LSSYEQVVND YFPVVQEQLT AHPTYKVIVT
  SP400   NFDLKEINDI CSGCRGHDGF TSSWRSVADT LRQKVEDAVR EHPDYRVVFT
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  SP400   GHSLGGALAT VAGADLRGN. .GY...DIDV FSYGAPRVGN RAFAEFLTVQ
    Pcl   GHSLGAAVAT LAATDLRGK. .GYP..SAKL YAYASPRVGN AALAKYITAQ
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          201                                                250
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  rhidl   G.IPFQRTVH KRDIVPHVPP QSFGFLHPGV ESWIKS.GT. ......SNVQ
  SP400   TGGTLYRITH TNDIVPRLPP REFGYSHSSP EYWIKS.GTL V.PVTRNDIV
    Pcl   G..NNFRFTH TNDPVPKLPL LSMGYVHVSP EYWITS.PNN A.TVSTSDIK
FoLnp11   A.GGEYRVTH ADDPVPRLPP LIFGYRHTTP EFWLSGGGGD KVDYTISDVK

          251                                                300
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  rhidl   ICTSEIETKD CSNSIVPFT. .SILDHLSYF DINEGSCL-- ----------
  SP400   KIEGID.ATG GNNQP.NIP. .DIPAHLWYF .GLIGTCL-- ----------
    Pcl   VIDGDV.SFD GNTGTGLPLL TDFEAHIWYF .VQVDAGKGP GLPFKRV---
FoLnp11   VCEGAA.NLG CNGGT.LGL. .DIAAHLHYF .QATDACNAG GFSWRRYRSA

          301                                          338
  rhimi   ---------- ---------- ---------- ------...
  rhidl   ---------- ---------- ---------- ------...
  SP400   ---------- ---------- ---------- ------...
    Pcl   ---------- ---------- ---------- ------...
FoLnp11   ESVDKRATMT DAELEKKLNS YVQMDKEYVK NNQARS..
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**Fig. 1**

**Alignment of lipase sequences**

SEQUENCE LISTING

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accagcgtta caggcgtcag tc 82

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00664

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 9/20 // C11D 3/386, A21D 8/04, A23L 1/105
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Oils-fats-lipids 1995. Proceedings of the World congress of International society for fat research. 21st. Oct. 1995, Vol. 1, Atomi H. et al: "Microbial Lipases-from Screening to Design", page 48 - page 51 | 1,6-17,21, 25-34,38-50, 53-63 |
| X | Protein Science, Volume 8, 1999, Holger Scheib et al, "Stereoselectivity of Mucorales lipases toward triradylglycerols- A simple solution to a complex problem", page 215 - page 221, see abstract, page 217 | 1,6-17,21, 25-34,38-50, 53-63 |
| X | WO 9205249 A1 (NOVO NORDISK A/S), 2 April 1992 (02.04.92), see abstract, page 2, lines 7-20, page 3, lines 12-14, lines 25-30, page 4, lines 1-18 | 1,6-17,21, 25-34,38-50, 53-63 |

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 8 May 2000 | 09 -05- 2000 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Yvonne Siösteen/EÖ<br>Telephone No. +46 8 782 25 00 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00664

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9401541 A1 (NOVO NORDISK A/S), 20 January 1994 (20.01.94), see abstract, page 2, lines 19-24, page 5, lines 29-32 | 1,6-17,21, 25-34,38-50, 53-63 |
| | -- | |
| A | Croatica Chemica Acta, Volume 68, No 3, 1995, Peter Stadler et al, "Understanding Lipase Action and Selectivity. Conference Paper" page 649 - page 674 | 1,6-17,21, 25-34,38-50, 53-63 |
| | -- | |
| A | Jaocs, Volume 75, No 6, 1998, Mohamed M. Soumanou et al, "Two-Step Enzymatic Reaction for the synthesis of Pure Structured Triacylglycerides" page 703 - page 710 | 1,6-17,21, 25-34,38-50, 53-63 |
| | -- | |
| A | ISSN 1392-0146 Biologija. 1995. Nr. 1-2, I. Bachmatova et al: "Lipase of Pseudomonas mendocina 3121-1 and its Substrate Specificity", page 57 - page 58 | 1,6-17,21, 25-34,38-50, 53-63 |
| | -- | |
| | -------- | |

## INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00664

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).:

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Claim 1, part of 25-34, part of 38-50 and part of 55-63

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00664

The international Searching Authority considers that the present application contains at least seven inventions.
The observation is based on the following reasons:

The inventions listed do not relate to a single inventive concept under PCT Rule 13.1 because they lack the same or corresponding special technical feature for the following reasons:

What could be seen as a unifying feature of the claims is a method for producing lipase variants with altered activity on ester bonds of the substate.

The prior art discloses methods for producing lipase variants with altered substrate specificity on ester bonds of the substrate. These methods are based on the knowledge of the 3-dimensional structure of lipase. See e.g Atomi et al, Oils-Fats-Lipids 1995, Proc. World Congr.Int Soc. Fat Res., 21st , 1996, Meeting Date 1995, Volume 1, 49-50, which discloses mutated lipases with altered specificity towards long and short chain fatty acid Me esters. It is also known from many patent documents to make lipase variants with altered catalytic activity –see e.g. WO 92/05249 and WO 94/01541.

Unity of invention exists only when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding "special technical feature"- i.e. features that define a contribution which each of the inventions make over prior art. (See Annex B to administrative Instructions and Rule 13.1)

Therefore the technical feature being a method of producing lipase variants with altered activity on the ester bond of the substrate is not a feature that define a contribution over prior art.

Consequently, the different methods of producing lipase variants cannot be seen as a unifying novel special technical feature.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00664

The claims consist of at least the following six inventions:

1) A method of producing a lipolytic enzyme variant by altering an amino acid within 10Å of the C atom at the sn2 position of the glycerol part of a substrate triglyceride in a three-dimensional structure of the parent lipolytic enzyme and substrate. See claim 1. The first invention claimed also relates to lipolytic enzyme variants being mutated in the region defined in claim 1 and having an altered activity on the selected ester bond. See claim 21 and part of the following claims; claims 6-17,25-34,38-50 and 53-63.

2) A method of producing a lipolytic enzyme variant by altering an amino acid in the catalytic triad of the parent enzyme. See claim 2.

3) A method of producing a lipolytic enzyme variant by altering an amino acid situated at the C-terminal of an active His residue.See claim 3.

4) A method of producing a lipolytic enzyme variant by altering an amino acid among 10 amino acid residues at the C-terminal. See claim 4.

5) A method of producing a lipolytic enzyme variant by altering an amino acid in the lid.See claim 5.

6) A method of preparing a lipolytic enzyme variant for use in baking, characterized by screening for enzyme variants having a) a higher ratio selectivity for long-chain fatty acyl groups, b) a higher activity on digalactosyl diglyceride and a higher phospholipase activity.See claims 18-20.

Since, however claims 2 –3 and claims 4-5 could be treated for one extra fee for each pair of invention , the applicant is only invited to pay for three extra fees in total.

Therefore, although the claims are composed of at least six different inventions, the applicant is invited to pay only three extra fee as the rest of the claims could be searched without effort justifying six additional fees.

Due to the very complex construction of the claims and the fact that the search so far has not covered all aspects of the invention, it may be that further non-unity remarks can appear.

The search has been restricted to the first invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00664

Several of the claims are constructed in such a complex way and being so broad and speculative that it is not possible to carry out a meningful complete search.See Art 17(2)(a). Some examples of such claims are:

Claims 7-8 and 27-34 which are too broadly formulated and relates to a large number of variable positions in the amino acid chain without being linked to one of the specific regions of claims 1-4. Therefor it will not bee possible to perform a meaningful search for the whole scope of these claims.

Claims 10,14,15 and 17 which represent some of the multiple dependent claims of the application being too unclear because they are linked to ***any*** of the specific region of claims 1-5. It will not considered possible to permit a meaningful search for the whole scope of the claims. See Rule 6.4(a).

Claims 35-50 which relate to lipolytic enzymes where the sites for alterations are specified as the positions ***corresponding*** to specific positions in the Humicola lanuginosa lipase are too indefinite to be searched for.

Patent claims taken singly as well as in totality, must be clear and concise (PCT Article 6) in order to enable potential users to ascertain, ***without undue burden***, the scope of the protection.

The search of the first invention has been restricted to a method of producing lipase variants where amino acids have been altered in the region specified in claim one. The search also covers lipase variants produced by the method of claim one.

## INTERNATIONAL SEARCH REPORT

Information on patent family members

02/12/99

International application No.
PCT/DK 99/00664

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9205249 A1 | 02/04/92 | AT 169671 T | 15/08/98 |
| | | AU 657278 B | 09/03/95 |
| | | AU 8617291 A | 15/04/92 |
| | | CA 2092615 A | 14/03/92 |
| | | DE 69129988 D,T | 18/03/99 |
| | | DK 219490 D | 00/00/00 |
| | | EP 0548228 A,B | 30/06/93 |
| | | SE 0548228 T3 | |
| | | ES 2121786 T | 16/12/98 |
| | | FI 931124 A | 12/05/93 |
| | | JP 6501153 T | 10/02/94 |
| | | US 5869438 A | 09/02/99 |
| | | US 5892013 A | 06/04/99 |
| | | DK 219590 D | 00/00/00 |
| | | DK 219690 D | 00/00/00 |
| WO 9401541 A1 | 20/01/94 | BR 9306694 A | 08/12/98 |
| | | EP 0652945 A | 17/05/95 |
| | | JP 7508416 T | 21/09/95 |
| | | MX 9304027 A | 31/05/94 |